ASSESSMENT OF THE POTENTIAL OF FUNGAL SPECIES IN THE BIOACCUMULATION OF LEAD, NICKEL AND CADMIUM FROM REFINERY EFFLUENT

BY

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NIGERIA

APRIL, 2015

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A THESIS SUBMITTED TOTHE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIALFULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OFMASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

APRIL, 2015

DECLARATION

I hereby declare that the work presented in this thesis entitled**"Assessment of the potential of fungal species in the bioaccumulation of lead, nickel and cadmium from refinery effluent"** was carried out by me in the Department of Microbiology, Ahmadu Bello University Zaria, Kaduna State, Nigeria, under the supervision of Dr. D.A. Machido and Prof. S.E. Yakubu. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Blessing Amaka EZEONUEGBU _____ _____

M.Sc/Sci/10041/2011-2012 **Signature** **Date**

CERTIFICATION

This thesis entitled: "ASSESSMENT OF THE POTENTIAL OF FUNGAL SPECIES IN THE BIOACCUMULATION OF LEAD, NICKEL AND CADMIUM FROM REFINERY EFFLUENT" by Blessing AmakaEZEONUEGBU, meets the regulations governing the award of Masters of science degree in Microbiology of Ahmadu Bello University Zaria, and is approved for its' contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to **God the Father**, **God the Son** and **God the Holy Spirit,** for His lamp unto myfeet and His light unto my path. To Him alone be all the glory.

ACKNOWLEDGEMENTS

I wish to express my special appreciation and thanks to my honorable supervisors, Dr. D.A. Machido and Prof. S.E. Yakubu. You have been a tremendous father and a mentor to me. I wish to thank you for your time, priceless effort, sleepless nights, encouragement and illuminating views tothis research work.

Special thanks go to my family. Words cannot express how grateful I am to my parents Chief and Lolo BenaiahUmeagudosiEzeonuegbu. Thank you for all your sacrifice, support and prayers which brought me this far. I wish to thank my siblings who supported and incented me to strive towards my goals.

My sincere thanks also go to Mr. Shittu Adamu, Head of Microbiology Laboratory and his assistants; Mr. DanlamiDalhatu and Mr. Sani Jumaare for assisting me relentlessly till the end of this work. I appreciate the support and encouragement given to me by AlhajiAliyuGwadabe (Baba). Thank you for giving me all I needed from Microbiology departmental store.

I am grateful to the staff of Kaduna Refinery and Petrochemical Company (KRPC). My special thanks go to Mrs Success Ujuji of the Department of Health, Safety and Environment of KRPC. Thank you for your support and help in taking me around for my sample collection.

I wish to specially thank my lecturers, friends and coursemates. Thank youfor your encouragement and love as a family. God bless every one of you.

ABSTRACT

This study was carried out to assess the potential of fungal species in the bioaccumulation of lead, nickel and cadmium by fungal species isolated from refinery effluent and effluent impacted River. Physicochemical, heavy metal and mycological analyses were carried out on untreated and treated effluent from Kaduna Refinery and Petrochemical Company (KRPC) and water samples from Romi River, Kaduna State, Nigeria, using standard procedures. A total of 133 fungal isolates belonging to 15 species (*Aspergillus flavus, A. niger, A. fumigatus, A. carbonarius, A. glaucus, Penicillium* spp., *Fusarium* spp., *Curvularia* spp., *Trichoderma*spp., *Nigrospora*spp., *Microsporum* spp., *Rhizoctonia* spp., *Trichophyton*spp., *Geotrichum* spp.and *Chaetophoma* spp.) were isolated from the study site, identified and characterized on the basis of their macroscopic and microscopic morphologies using standard taxonomic guides. *Aspergillus flavus* had the highest percentage occurrence of 18.05% followed by *Penicillium* spp. and *Curvularia* spp. which had equal occurrence of 12.78% each. *A. glaucus* and *Trychophyton*spp. had the least and equal percentage occurrence of 0.75% each. The isolates were inoculated into duplicate 100ml flask containing 50ml of potato dextrose broth (PDB) supplemented with 5,10 and 15µg/ml of Pb, Ni, and Cd analytical grade salts. Each test isolate was inoculated into duplicate flask containing the same medium without the heavy metals to serve as control. All inoculated flasks were incubated aerobically at room temperature on a rotator shaker for 7days. The mycelial mats were harvested by filtering the cultures through pre weighed filter paper (No.1). The filters bearing the mycelial mats were dried in an oven at 70^{o}C for 18hours and weighed. Yields of dry mycelial mats in the heavy metal supplemented medium were also comparable to those grown in heavy metal free PDB medium. It was observed that 52 out of the 133 fungal isolates tested, resisted and grew in the medium containing 5,

10 and 15ppm of the test heavy metals. pH and temperature optimization studies on growth conditions of the resistant isolates were carried out at varying pH of 4, 5, 6 and 7 and varying temperatures of 25^{o}C, 30^{o}C and 35^{o}C. The various test isolates grew optimally at the varying pH and temperature levels except pH 7 and temperature of 35^{o}C. Heavy metal uptake capacity and removal from refinery effluent by the test fungal isolates were also determined. The genera *Aspergillus, Penicillium, Trichoderma, Curvularia, Fusarium* and *Nigrospora*accumulated and removed substantial amount of Pb, Ni and Cd from the effluent. *Trichoderma* sp. and *Microsporum*sp. had an uptake capacity of 0.28mg/g of lead each. *Nigrospora*sp. and *Fusarium* sp. had an uptake capacity of 0.14mg/g and 0.13mg/g of nickel respectively. The uptake capacity of cadmium by *Penicillium* sp. was 0.02mg/g while *A. flavus*and *A. niger* removed 71.29% each of lead. *A. niger*was able toremove 85.06% of nickel while 85% of cadmium was removed by *Penicillium* sp. It was therefore concluded that, fungi constitute a significant proportion of the microflora of sites contaminated with the refinery effluent and could be playing an important role in the remediation of sites receiving the effluent. Also, their tolerance to Pb, Ni and Cd indicates that these test fungal isolates, could be employed in the treatment of refinery effluents and bioremediation of water bodies and sites polluted with Pb, Ni and Cd.

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CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

One of the most critical problems of developing countries is improper disposal of vast amount of wastes generated by anthropogenic and industrial activities (Achi and Kanu, 2011). The disposal of these wastes into the ambient environment especially fresh water reservoir has rendered the recipient bodies of water unsuitable for both primary and or secondary use. The discharge of industrial effluents into receiving water bodies in Nigeria invariably results in the presence of high concentrations of pollutants in the water and bottom sediment.

Beddri and Ismail (2007), reported that waste water effluents from petroleum refinery and petrochemical plants contain a diverse range of pollutants including heavy metals, oil and grease and have high biological oxygen demand (BOD) - bearing materials, suspended solids, dissolved solids, phenols and sulfides. Heavy metals in refinery effluent mainly originate from the feedstock. Others are from corrosion products of the equipment and pipes, process chemical additives and from materials like catalysts and other chemicals used in processes downstream of the primary distillation. The most common of these metals are nickel, vanadium, copper, chromium, lead, cadmium, zinc and selenium. Mercury has also been found to appear as impurity in natural gas and crude oil (Namazi *et al.*, 2008; Alao *et al*., 2010; Nabi *et al.*, 2010).

Heavy metals, particularly lead, chromium, arsenic and mercury are environmental pollutants threatening the health of human population and natural ecosystem through direct ingestion, inhalation and consumption of contaminated fish, animals or plants (Alao *et al*., 2010). However some metals (cobalt (Co), copper (Cu), iron (Fe),

Potassium (K), magnesium (Mg), Sodium (Na), Nickel (Ni), and Zinc (Zn)) are essential, serve as micronutrients and are used for redox- processes, to stabilize molecules through electrostatic interactions as components of various enzymes and regulation of osmotic pressure (Subudhi and Kar, 2008). While many other metals (gold, aluminum, cadmium, silver, lead and mercury) have no biological role and are non-essential, they are potentially toxic to living organisms (Nimalkumar *et al*., 2010; Burgess and Almeida, 2012). Toxicity of non-essential metals occurs through the displacement of essential metals from their natural binding site or through ligand interactions. The accumulation of these metals in water depends on many factors such as the type of industries in the region, people's way of life and awareness of the impact done to the environment by careless disposal of wastes.

Several methods have been used for the treatment and removal of heavy metals in contaminated sites. Conventional and physicochemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu *et al*., 2002) for heavy metal removal are economically expensive and have disadvantages like incomplete metal removal, high demands of reagents and high energy requirements and generation of toxic sludge or other waste products that require careful disposal (Sun and Shao, 2007; Hemambika *et al*., 2011). With the increase in environmental awareness and legal constraints being imposed on discharge of effluents, the need for alternative cost effective technologies is essential. In this endeavor, biological approach has a great potential for the achievement of this goal and it is economical. Microbial populations in metal polluted environment adapt to toxic concentration of heavy metals and become metal resistant (Prasenjit and Sumathi, 2005). The response of microorganisms towards toxic heavy metals is of important interest in the reclamation of polluted sites (Shaukar *et al*., 2007).

Biosorption and Bioaccumulation are biological methods of environmental control of heavy metal pollution. These processes involve metal uptake by either non- living (biosorption) or living (bioaccumulation) biomass (Volesky, 2001; Chojnacka, *et al.*, 2007). Biosorption is influenced by environmental factors like pH, temperature, ion competition, variations in the chemical composition of the cell wall, capsule, and polysaccharides (when released). In any case, metals can be deposited around the cell as phosphate, sulphate and oxides (Giovanni, 2009). These biological processes could be an effective solution to the majority of the problems associated with heavy metals released in the environment by industries (Giovanni, 2009). They offer many advantages over conventional methods including cost effectiveness, efficiency, minimization of chemicals and biological sludge, requirement of additional nutrients and regeneration of biosorbents with possibility of metal recovery (Ronda *et al*., 2007). Microorganisms are nature's original recyclers, converting toxic organic compounds to harmless products which are often carbon dioxide and water. Previous studies reported that scientists are exploring microbial and associate biota within the ecosystem to degrade, accumulate, and or remove the pollutants from the environment (Paknikar *et al*., 2003; Gadd, 1993a; Khan and Khoo, 2000).

Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake (Gupta *et al*., 2000; Hemambika *et al.*, 2011). These mechanisms result from complexation and or ion exchange reactions between metal ions and charged chemicals of their cell walls (Gupta *et al*., 2000; Hemambika *et al.*, 2011). Fungi are widely distributed from the artic to the tropics. They are capable of metabolizing enormous variety of substances because of large number of diversified enzymes they produce and have become tools for the biochemists, chemical engineers, physiologists, biophysicists and geneticists

(Angumeenal and Venkappayya, 2004). They accumulate micronutrients such as copper, zinc, manganese and toxic metals like nickel, cadmium, tin, lead and mercury in amount higher than nutritional requirement (Angumeenal and Venkappayya, 2004). Studies have shown that the biomass from fungi such as *Phanerochaete chrysosporium* (Pal and Vimala, 2011), *Polyporus squamosus* (Wuyep *et al.,* 2007) *Aspergillus, penicillium, Trichoderma* species(Tereshina *et al*., 1999; Thipeswamy, 2012) and *Rhizopus arhizus* (Tapan *et al.,* 2010) had been found capable of removing heavy metals. In the light of the above, the present study is aimed at investigating the metal removal and bioaccumulation potential of fungi from refinery effluent.

1.2 Statement of Problems

Heavy metals have been shown to pose significant problems to human health. They contaminate the main food resources, the environment and drinking water. Also excess loading of hazardous wastes has led to scarcity of clean water and pollution of soil, thus limiting crop production (Karmaludeen *et al*., 2003). Metals may be accumulated, concentrated and magnified within food chains, causing higher trophic organisms to become contaminated with high concentrations of chemical pollutants and metal contaminants than their prey (Lee *et al*., 2000; Hargrave *et al.,* 2000). This situation is mainly ascribable to the production cycle involving heavy metals, industrial sectors like mechanical, electronic, tanning, galvanic, mining and oil industry (WHO, 2006). According to Achi and Kanu (2011), the major industrial categories in Nigeria are metals and mining, food and beverages, tobacco, breweries, textile and petroleum refinery. Effluents from these industries contain toxic and hazardous materials that settle in rivers as part of the bottom sediment. They pose health hazards to the urban population of the people that depend on the water as source of supply for domestic uses (Rahman *et al*., 2008). According to Emoyan *et al*., (2006) and Idise *et al*. (2010),

petroleum refining produces large amounts of effluents that are toxic. Accidental discharge of crude petroleum, field oil and grease from the refinery also contributes to the suppression of plants and animals life (Okerentugba and Ezeronye, 2003; Potin *et al.*, 2004).

According to WHO (2006), the metals of most immediate concern are lead, cadmium, chromium, cobalt, copper, nickel, mercury and zinc which are included in the "priority pollutants" due to their high toxicity. Heavy metals like these are immobilized and carried into the food web as a result of leaching from waste dumps, polluted soil and water. The metals increase in concentration at every level of the food chain and are passed into the next higher level of the chain by a phenomenon called biomagnification (Paknikar *et al.*, 2003). For example, nickel can cause asthma, skin rashes and cancer. Copper, nickel, zinc and selenium are essential micronutrients which can become toxic at high concentrations. Millions of children have suffered ill health due to lead (Rao *et al.*, 2005; Thipeswamy *et al.*, 2012). Mercury is problematic in cryogenic petrochemical facilities and is toxic to humans. Chromium can cause nasal problems, ulcers as well as kidney and liver damage and is also carcinogenic. Cadmium can cause damage to cell membranes and the presence of heavy metals in aquatic environment can cause several damages to aquatic life (Thipeswamy *et al.*, 2012). Therefore, a study like this, to determine whether or not heavy metals can be accumulated in the fungus species would give an insight into the possibility of using species of fungus for their removal from the environment.

1.3 Justification

Environmental pollution with heavy metal is a global concern being everywhere, to different degree and specific to certain part of the biogeosphere. At a global scale approximately 2.4 million tons of liquid effluents containing heavy metals and other

toxic materials are generated per annum (Veglio and Beolchini, 1997; Burgess and Almeida, 2012).

Heavy metals are of critical concern to human health and environmental issues due to high occurrence in soluble form extremely toxic to biological systems and their classification as carcinogens and mutagens (Alloway, 1995; Hemambika *et al*., 2011). To avoid health hazards, it is important to remove these toxic heavy metals from waste water before its disposal.

Bioremediation through the use of Biosorption and bioaccumulation approach is an emerging form of technology in which microbes including fungi are used to clean up contaminated soil and water and to remove or stabilize the contaminants (Veglio and Beolchini, 1997; Strong and Burgess, 2008). Many microbial population including fungi have been identified as superior candidates for metal bioremediation and major advantages of fungi are their significant metal uptake ability at low anticipated price (Bai and Abraham, 2001). The cell wall of fungi present a multi-laminated architecture where up to 90% of the dry mass consist of amino and non-amino polysaccharides and proteins which offer many functional groups (such as carboxyl, hydroxyl, sulphate, phosphate and amino groups) for binding metal ions (Murugesan and Maheswari, 2011). Furthermore, fungal mycelia penetrate oil and increase the surface area available for degradation by other microbes. Fungi are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited (Obire and Putheti, 2009). Finally, fungi are easy to transport, genetically engineered and produce in large quantity (Obire and Putheti, 2009). Judging from the deleterious effects heavy metals have on health and environment, bioaccumulation could be valuable in removing these effects and ensure safe environment.

1.4 Aim

The aim of this study was to assess the potentials of fungal species in the bioaccumulation of lead, nickel and cadmium from refinery effluent.

1.5 Specific Objectives

The specific objectives of this study were

1. To determine the physicochemical properties of the refinery effluent and receiving water body.
2. To isolate and characterize fungi from the refinery effluent and the receiving water body.
3. To establish whether or not the fungal isolates can resist the heavy metals (Pb, Ni and Cd).
4. To determine the optimum pH and temperature conditions of growth of the resistant isolates.
5. To determine the possible accumulation and removal of the heavy metals by the resistant fungal isolates in laboratory experiment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fungi

Fungi are members of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms. Fungi have a profound impact on the global ecosystems. They modify habitats and are essential for many ecosystem functions (Le Calvez *et al.,* 2009). Fungi form soil, recycle nutrients, decay wood, enhance plant growth and cull plants from the environment. The kingdom Fungi are divided into five phyla namely; Chytridomycota, Zygomycota, Basidiomycota, Ascomycota and Deuteromycota (Prescott *et al.,* 1999; Buckley *et al.,* 2007).

2.1.1 Phylum Chytridiomycota

"Chytrids" are a small group of fungi with approximately 900 identified species occurring in a wide range of aquatic and terrestrial habitats around the world. The feature that is shared by all members of this phylum is the formation of zoospores with a posterior whiplash flagellum. Most chytrids are saprotrophs using substrates such as cellulose, chitin, and keratin as a food source. But, some chytrids are economically important plant pathogens and vector of plant viruses. Examples are *Synchytrium endobioticum, Batrachochytrium dendrobatidis* and *Olpidium* (Alcamo and Pommerville, 2004; Parniske, 2008).

2.1.2 Phylum Zygomycota

The most commonly encountered Zygomycetes are members of orders Mortierellales and Mucorales. Many members of these two orders are saprotrophs with rapidly growing, coenocytic mycelium. Their sexual reproductive state is the zygospore,and

many of these fungi produce a large number of readily dispersed asexual spores called sporangiospores. Members of order Mucorales, commonly called mucoraceous fungi, are common in soil, dung, plant material, and other types of organic matter. Some mucoraceous fungi are plant or animal pathogens, and others are used in the production of Asian foods such as tempeh. Species of *Mucor* and *Rhizopu*s are commonly isolated from decaying organic matter and can cause decay diseases of fleshy fruits, and vegetables (Prescott *et al.,* 1999).

2.1.3 Phylum Ascomycota

Phylum Ascomyta is the largest group of fungi, with approximately 33,000 described species in three subphyla—Taphrinomycotina, Saccharomycotina, and Pezizomycotina. Members of this phylum reproduce sexually or meiotically via production of ascospores inside a sac-like structure called an ascus. Many species of Ascomycota also (or exclusively) produce spores through an asexual or mitotic process; these spores, called conidia, exhibit a wide range of size, shape, color and septation among the different fungi in which they are formed. Examples are *Schizosaccharomyces, Sclerotinia* and *Monosporascus* (Parniske, 2008).

2.1.4 Phylum Basidiomycota

Members of phylum Basidiomycota produce basidiospores on a typically club-shaped structure called a basidium. Characteristic of the mycelium of many members of Basidiomycota is the presence of clamp connections and doliporesepta. Three main lineages are recognized in phylum Basidiomycota are subphyla Ustilaginomycotina, Pucciniomycotina, and Agaricomycotina. Ustilaginomycotina and Pucciniomycotina are composed mostly of plant parasitic species; known as smut and rustfungi, respectively, characterized by a state that produces thick-walled teliospores. Members of

Ustilaginomycotina are species of *Tilletia* and *Ustilago*. *Ustilagomaydis*. Subphylum Pucciniomycotina include the group of plant parasites called rust fungi (Buckley, 2007). The rust fungi are remarkable in having as many as five distinct types of spores in a single life cycle (spermatia, aeciospores, urediniospores, teliospores, and basidiospores). Rust fungi that produce all five spore states are *macrocyclic*, those that do not form uredinospores are *demicyclic*, and those that do not form urediniospores and aeciospores are *microcyclic* (Chang and Miles, 2004).

Subphylum Agaricomycotina, previously known as the Hymenomycetes, includes the morphologically diverse group of fungi that produce basidia in various types of fruiting bodies. This group includes the fungi commonly known as mushrooms, puffballs, shelf fungi, stinkhorns, jelly fungi and bird's nest fungi. Many species are saprotrophic, utilizing dead plant material including woody substrates (Buckley *et al.*, 2007). Some of these saprotrophic species are cultivated for food, for example, the common button mushroom (*Agaricus bisporus*) and oyster mushrooms (*Pleurotus ostreatus*).

2.1.5 Phylum Deuteromycota

The division Deuteromycota or the Fungi imperfecti contains fungi that lack a sexual phase (perfect stage). Most fungi imperfecti are terrestrial, with only a few being reported from fresh water and marine habitats. The majority are either saprophytes or parasites of plants. A few are parasitic on other fungi (Prescott *et al.*, 1999). Reproduction of Deuteromycota is strictly asexual, occurring mainly by production of asexual conidiospores. Genetic recombination is known to take place between the different nuclei. Imperfect fungi have a large impact on everyday human life. The food industry relies on them for ripening some cheeses. The blue veins in Roquefort cheese and the white crust on Camembert are the result of fungal growth. The pharmaceutical

industries rely on them for antibiotic and secondary metabolite (Huang *et al.*, 2008). They are promising tools for bioremediation of polluted environment (Huang *et al.*, 2008). Examples are *Penicillium, Aspergillus, Alternaria, Fusarium, Rhizoctonia,* and *Verticillium.*

2.2 Heavy Metals

Heavy metals are defined as elements with atomic number greater than 22 and a density greater than 5g/ml (Elen *et al.*, 2011). They are not only widespread pollutants of great concern but are non-degradable and persistent. Common sources of heavy metal pollution in the environment include discharge from industries such as refining electroplating, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes (Zouboulis *et al*., 2004).These metals are used in various industries from which effluents are discharged into the environment which could cause numerous modifications of microbial communities, activities and highly toxic to human even at low concentration (Chen and Wang, 2008; Elen *et al.*, 2011). Metal ions can be classified as; essential, toxic, radionuclides and semi metals (Chen and Wang, 2007).

2.2.1 Essential metals

Essential metals are generally important for human health and metabolism. These metals are so called because they form an integral part of metabolic and biochemical process, provide vital co factors for metalloproteins and enzymes, serve as catalyst necessary for cellular function and regulate osmotic pressure (Doelman *et al*., 1994; Bruins *et al*., 2000). They are widely found in nature and soil as mineral deposits. Examples of such metals are sodium, potassium, magnesium, molybdenum, nickel, calcium and zinc. A characteristic associated with essential metals is that the body

provides homeostatic mechanism that increases or decreases their uptake and excretion as needed to maintain their levels in the body. Although essential metals are necessary, they become toxic at high concentration (Bruins *et al*., 2000).

2.2.2 Toxic heavy metals

Toxic metals comprise non-essential metal ions with no known biological function in the body and highly toxic and harmful to microorganisms. Examples are lead, arsenic, silver, nickel silicon, aluminum, titanium and mercury. At high concentrations, however, these heavy metal ions form unspecific complex compounds, which lead to toxic effects. For example in ectotoxicology terms, hexavalent forms of mercury, chromium, lead and cadmium ions are the most dangerous (Elen *et al.*, 2011).

2.2.3 Radionuclides

These are radioactive isotopes with unstable nucleus characterized by the number of proton and neutrons in the nucleus as well as the amount of energy contained within an atom. Radionuclides are important in nuclear medicine procedures but are toxic to cells. Examples are uranium, radium, thallium (Chen and Wang, 2007).

2.2.4 Semi- metals

These are metalloids that share some properties of metals and some of the properties of non-metal and are present in the environment in cationic and anionic forms (Chen and Wang, 2007). The reactivity of metalloids depends on the element with which they are reacting with. For example, boron acts as a non-metal when reacting with sodium yet as a metal when reacting with fluorine. Other examples of semi metals are silicon, arsenic, polonium, tin, selenium.

2.3 Industrial Wastes

Industrial wastes are solid, semi-solid, liquid, or gaseous, unwanted or residualmaterials from an industrial operation such as refining, manufacturing, mining or agriculture. Various wastes are produced at every production stage depending on the type of industry, the raw materials used and the type of technology utilized (Agoyi, 2002). These wastes are not safely treated due to lack of highly efficient and economic technology. Industrial wastes contain various toxic metals, harmful gases, and several organic and inorganic compounds. The discharge of these toxic wastes or effluents has led to a major loss in the ecological, social and economic perspective of the environment becomes disastrous to surface water, ground water and soil, as well as cause fatal diseases like cancer, delayed nervous responses, mutagenic changes and neurological disorders (Wahaab, 2000; Balaji *et al.*, 2005).

2.3.1 Characteristics of industrial waste

The characteristics of industrial waste vary, depending on the type of industry but are broadly classified as follows (Agoyi, 2002):

a. Organic substances that deplete oxygen contents of the receiving water body.
b. Inorganic substances like chloride, nitrogen, carbonates that render the water body unfit for further use and encourage undesirable growth of micro-plants in the water body.
c. Heavy metals and toxic substances like mercury, lead, nickel, cadmium, cyanide, acetone, sulphate and alcohol which can cause damage to fauna and flora of the receiving water body.
d. Oil and grease and other floating substances which render the stream unpleasant to the eyes and interfere with self-purification of the stream.

e. Acid and alkali, which make the receiving stream unsuitable for growth of fish and other aquatic life.
f. Colour producing substances like dyes from dying units which are aesthetically objectionable when present in receiving water body.

2.3.2 Sources of industrial wastes

Common sources of environmental pollution include discharge from industries such as electroplating, refining, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes (Zouboulis *et al*., 2004). Materials and contaminants generated by these industries have been reported and are summarized in Table 2.1 (Ho *et al.*, 2014).

2.3.3 The effects of industrial wastes to freshwater

The quality of freshwater is very important as it is highly consumed by human for drinking, bathing, irrigation and recreational purposes. Contaminants from industrial discharge flow into this fresh water (rivers and groundwater) which may reduce the yield of crops and the growth of plant and also affect aquatic organisms and human health.

2.3.3.1 Impact of industrial waste on river

River, including its main course and tributaries, is a large flow of water system which carries load of dissolved and particulate matters from both natural and anthropogenic sources along with aquatic lives and other contents. These substances as they move downstream, experience chemical and biological changes. Thus, the water chemistry of a river is affected by the lithology of the reservoir, atmospheric, and anthropogenic inputs. Furthermore, the transport of natural and anthropogenic sources to the oceans

and their state during land–sea interaction can be determined by the water quality from rivers and estuaries which could bring the understanding on the fate of metals, organic and inorganic matters along the river to the ocean (Ho *et al.*, 2014).

2.3.3.2 Impact of industrial waste on groundwater

Groundwater is regarded as the largest reservoir of drinkable water for mankind. To many countries, groundwater is one of the major sources of water supply for domestic, industrial and agricultural sectors. Human activities like industrialization are responsible for groundwater contamination. It is estimated that approximately one-third of the world's population are using groundwater for drinking purposes. Pollution of ground water due to industrial effluents is a major issue (Vasanthavigar *et al.*, 2011). Poor groundwater quality brings negative impact to human health and plant growth. In developing countries, it is estimated that around 80% of all diseases are directly related to poor drinking water quality and unhygienic conditions (Olajire and Imeokparia, 2001; Vasanthavigar *et al.*, 2011).

2.4 Effluent Characteristics and Water Quality

Water pollution is commonly defined as any physical, chemical or biological change in water quality which adversely impacts on living organisms in the environment or which makes a water resource unsuitable for one or more of its beneficial uses. Some of the major categories of beneficial uses of water resources include public water supply, irrigation, recreation, industrial production and nature conservation. The nature of a pollutant and the quantity of it are important considerations in determining its environmental significance (Walakira, 2011). Parameters identifiable to effluent characteristics and its effect to water quality are biodegradable organic substances.

Table 2.1: Contaminants and Materials Generated by Industries

Type of Industry	Contaminants Generated	Reference
Refinery and Petrochemicals,	Metals: Cd, Cr, Cu, Iron (Fe), Ni, Pb, Zn, Al, Ba, Mo, Sr, Zn, Cu, Li, V, Ag, Co and Se	Shiozawa *et al*. (1999)
Textile, plastics, metal fabrications indutries	Organic/ inorganic matter and parameter: phosphate, ammonia,nitrate, sulphates, chloride, aluminium, benzene, styrene, toluene, indene, naphthalene, ethyl benzene, xylene, oil and grease, BOD, COD, electrical conductivity, pH, total alkalinity, total hardness, total organic carbon (TOC), total suspended solids (TSS) and total dissolved solids (TDS)	Ahmed *et al.* (2011)
Dyeing and printing industries	Metal: Cu, Fe, Zn and Mn Organic/inorganic matter and parameter: TDS, TSS, COD, BOD, chlorides, sulfates, carbonates and sodium, calcium and magnesium.	Singh *et al.* (2005)
Tanneries, paint production, paper mills, and metal processing plants	Metal: Fe, Mn, Pb, Zn, Cu, Ni, Cr, Cd , Co Organic/inorganic matter and parameter: DO, COD Chemicals, beverage	Jonathan *et al*. (2008)
Pharmaceutical and food industries	Metal: Fe, Zn, Cu, Ni, Pb, Cr	Radić *et al.*(2010)
Cystine production industry	Organic/ inorganic matter and parameter Sodium, chloride, calcium, COD, BOD	Kocan *et al.*(2001)

2.4.1 Biodegradable organic substances

Effluents from industries, processing plant, animal products as well as human and animal wastes contain a mixture of complex organic substances such as carbohydrates, proteins and fats as their major pollution load. These substances are readily biodegradable and when introduced into the environments are quickly decomposed through the action of natural microbial populations (Walakira, 2011).

When a biodegradable organic waste is discharged into an aquatic ecosystem such as a stream, estuary or lake, oxygen dissolved in the water is consumed due to the respiration of microorganisms that oxidize the organic matter (Walakira, 2011). The more biodegradable a waste is, the more rapid is the rate of its oxidation and the corresponding consumption of oxygen (Walakira, 2011). Because of this relationship and its significance to water quality (dissolved oxygen levels in the water), the organic content of waste waters is usually measured in terms of the amount of oxygen consumed during its oxidation, termed the Biochemical Oxygen Demand (BOD).

In an aquatic ecosystem, a greater number of species of organisms are supported when the dissolved oxygen (DO) concentration is high. Oxygen depletion due to waste discharge has the effect of increasing the numbers of decomposer organisms at the expense of others. When oxygen demand of a waste is so high it tends to eliminate all or most of the dissolved oxygen from a stretch of a water body. Depletion of dissolved oxygen leads to anaerobic conditions and a general decline in the ecological and aesthetic qualities of the water body (Meertens *et al.,* 1995). Not only does the water then become devoid of aerobic organisms, but anaerobic decomposition also results in the formation of a variety of foul smelling volatile organic acids and gases such as hydrogen sulphide, methane and mercaptans (certain organic sulphur compounds). The

stench from these can be quite unpleasant and is frequently the main cause of complaints from residents in the vicinity.

Chemical Oxygen Demand (COD) is the measure of the total quantity of oxygen required to oxidize all organic material into carbon dioxide and water. COD values are always greater than BOD values, but COD measurements can be made in a few hours while BOD measurements usually take five days (BOD_5).

2.4.2 Plant nutrients

The availability of plant nutrients, particularly phosphorus and nitrogen are important determinants of the biological productivity of aquatic ecosystems. Nutrient deficient aquatic environments (oligotrophic) and those rich in nutrients (eutrophic) naturally accumulate nutrients over time, derived from drainage and sediment run off from its catchments. Human activities also greatly accelerate nutrient enrichment of water bodies through a process called cultural eutrophication ((Nyanda, 2000). While in the long term, cultural eutrophication accelerates the natural successional progress of aquatic ecosystems towards a terrestrial system; in the short term problems arise due to cyclic occurrences of algal blooms and decay. In warm weather, nutrients stimulate rapid growth of algae and floating aquatic weeds which cause the water to become opaque and exhibits unpleasant tastes and odours (Katima and Masanja, 1994).

In the natural world phosphorus is never encountered in its pure form, but only as phosphate. Phosphorous is one of the key elements necessary for growth of plants and animals. Phosphorus in its pure form has a white colour. White phosphorus is the most dangerous form of phosphorus that is known (Mosley *et al.*, 2004). When white phosphorus occurs in nature, this can be a serious danger to human health because it is extremely poisonous. White phosphorus enters the environment when industries use it

to make other chemicals and when the military uses it as ammunition. Through discharge of wastewater, white phosphorus ends up in surface waters near the factories that use it.

2.4.3 Pathogenic organisms

Faecal pollution of water resources by untreated or improperly treated sewage is a major cause for the spread of water-borne diseases. To a lesser extent, disease-causing organisms may also be derived from animal rearing operations and food processing factories with inadequate wastewater treatment facilities. Human diseases such as cholera, typhoid, bacterial and amoebic dysentery, enteritis, polio and infectious hepatitis are caused by water-borne pathogens (Walakira, 2011).

2.4.4 Turbidity

Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted in straight lines through a water sample. Turbidity in water is caused by the presence of suspended matter such as clay, silt, finely divided organic and inorganic matter, plankton, and other microscopic organisms (Galadima, 2012). High turbidity levels affect fish feeding and growth. Light attenuation by suspended particles in water has two main types of environmental impact: reduced penetration into water of light for photosynthesis and reduced visual range of sighted animals and people. High turbidity also due to total suspended solids supports high numbers of foreign microbiota in the water body, accelerating microbial pollution (Smith and Davies-Calley, 2001).

2.4.5 Electrical conductivity (EC)

Electrical conductivity is a function of Total Dissolved Solids (TDS) known as ions concentration, which determines the quality of water (Tariq *et al*., 2006). Electric Conductivity or TDS is a measure of how much total salts (inorganic ions such as sodium, chloride, magnesium, and calcium) are present in the water. Discharge of wastewater with a high TDS level has adverse impact on aquatic life, render the receiving water unfit for drinking and domestic purposes, reduce crop yield if used for irrigation, and exacerbate corrosion in water networks (Nadia, 2006). Conductivity, because it is easily measured, can serve as an indicator of other water quality problems. If the conductivity of a stream suddenly increases, it indicates that there is a source of dissolved ions in the vicinity (Mosley *et al.*, 2004).

2.4.6 pH

pH is a measure of the acid balance of a solution and is defined as the negative of the logarithm to the base 10 of the hydrogen ion concentration. pH changes can change the ecological balance of the aquatic system and excessive acidity can result in the release of hydrogen sulfide. In waters with high algal concentrations, pH varies diurnally, reaching values as high as 10 during the day when algae are using carbon dioxide in photosynthesis. pH drops during the night when the algae respire and produce carbon dioxide (Salequzzaman *et al.*, 2008).

The pH of water affects the solubility of many toxic and nutritive chemicals; therefore, the availability of these substances to aquatic organisms is affected Water with a pH greater than 8.5 indicates that the water is hard. Most metals become more water soluble and more toxic with increase in acidity (Walakira, 2011). Toxicity of cyanides and sulfides also increases with a decrease in pH (increase in acidity). The content of toxic

forms of ammonia to the non-toxic form also depends on pH dynamics (Mosley *et al.*, 2004).

2.4.7 Heavy metals

Heavy metals are among the major toxic pollutants in surface water. These have been found to be a problem in streams attributed to catchments with factories dealing with tanning, smelting, welding, renovation, manufacture and disposal of car batteries, petroleum and oil. Heavy metals pose significant hazard to human health and the ecosystem and its toxicity forms compounds that can be toxic, carcinogenic or mutagenic even at low concentrations. These metals may have pernicious effects if taken in high quantity or if the usual mechanisms of elimination are impaired. Metal ion toxicity is determined by various factors such as reduction-oxidation potential, electronegativity and physicochemical characteristics of the metal ions (Krishna e*t al.*, 2012).

2.5 The Impact of Industrial Discharge to Human Health

The impact of industrial discharge to human health is the utmost important criteria to consider apart from its effect to surface water, groundwater, living organism and sediments. Metals, organic and inorganic matter from industries have potential risk to human health. The health hazards of industrial discharge to human health are discussed below:

2.5.1 Metals

2.5.1.1 Lead (Pb)

Acute effects of lead are inattention, hallucinations; delusions, poor memory, and irritability are symptoms of acute intoxication. Lead absorption in children may affect their development and also results in bone stores of lead. It is associated with

behavioural effects, encephalopathy, nephropathy, and plumbism, which is connected with toxicity of high levels of lead which interferes with many processes in blood and organs (Grazyna and Pawel, 2010). Lead is poisonous to the heart, kidney and nervous systems. The symptoms of lead poisoning come mainly from the nervous systems i.e. insomnia, delirium, hallucinations and convulsions. Other symptoms are abdominal pain, headache, kidney failure and encephalopathy. Plumbism when not cured may lead to death (Jordao *et al.*, 2002; Grazyna and Pawel, 2010).

2.5.1.2 Nickel (Ni)

Nickel is an essential element to both plant and human, but high exposure to this metal can lead to cancer in organs of the breathing system, cardiovascular and kidney diseases (Jordao *et al.*, 2002). Exposure to nickel and its compounds may result in the development of a dermatitis known as "nickel itch" in sensitized individuals. The first symptom is usually itching, which occurs up to 7 days before skin eruption occurs. The primary skin eruption is erythematous, or follicular, which may be followed by skin ulceration. Nickel sensitivity, once acquired, appears to persist indefinitely (Ho *et al.*, 2014).

2.5.1.3 Cadmium (Cd)

Cadmium is harmful to both human health and aquatic ecosystems. Cd is carcinogenic, embryotoxic, teratogenic, and mutagenic and may cause hyperglycemia, reduced immunopotency, and anemia, as it interferes with iron metabolism (Rehman and Sohail, 2010). Furthermore, Cd in the body can cause kidney and liver damages and deformation of bone structures as well as osteoporosis, osteomalacia and several lung diseases (Abbas *et al.*, 2008).

2.5.1.4 Chromium (Cr)

Chromium (III) is essential nutrient for animal and essential to ensure human and animal lipids' effective metabolism. It can cause skin ulcer, convulsions, kidney and liver damage and can generate all types of genetic effects in the intact cells and in the mammals in vivo (Kherici- Bousnoubra *et al.*, 2009). It has also been reported that intensive exposure to Cr compounds may lead to lung cancer in man (Ho *et al.*, 2014). Cr (VI) is the most toxic form of chromium and has equivalent toxicity to cyanides. Other health problems that are caused by chromium (VI) are nose irritation and bleeding, weakened immune system, alteration of genetic materials, respiratory problems, lung cancer and death (Jordao *et al.*, 2002, Ho *et al.*, 2014).

2.5.1.5 Mercury (Hg)

The toxicity of mercury lies on the fact that that this metal mainly exists in form of evaporating liquid. The vapour of mercury easily penetrates the skin, mucus membranes and respiratory system. Mercury being one of the strongest neurotoxins is devastating to the kidney, neural and endocrine system. Because of the pathway of entry into the body organs, the tongue, teeth and gum are also affected. More toxic than mercury are metallorganic compounds of mercury e.g. Dimethylmercury (Grazyna and Pawel, 2010). At very low concentration, Hg can permanently damage the human central nervous system (Rai and Tripathi, 2009). Inorganic and mercury through biological processes can be converted into methyl mercury (MeHg). MeHg is organic, toxic, and persistent (Wang *et al.*, 2004; Rai and Tripathi, 2009). Furthermore, MeHg can cross the placental barriers and lead to foetal brain damage (Rai and Tripathi, 2009).

2.5.1.6 Zinc (Zn)

Zinc is an essential element to human and plant (Jordao *et al.,* 2002). Recent studies indicated that Zn is also involved in bone formation (Chou *et al.,* 2013; Jun *et al.,* 2015). According to Jun *et al.* (2015), Zinc is an essential trace element, which has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption *in vitro*. However, elevated intake of Zn can cause muscular pain and intestinal haemorrhage (Honda *et al.,* 1997; Jordao *et al.,* 2002).

2.5.1.7 Aluminum (Al)

High concentrations of Al can cause hazard to brain function such as memory damage and convulsion. In addition, there are studies which suggested that Al is linked to the Alzheimer disease (Jordao *et al.,* 2002; Tomljenovic, 2011; Mercola, 2014). Aluminum exposure is a factor in many neurological diseases, including dermentia, autism and Parkinson's disease (Mercola, 2014).

2.5.1.8 Iron (Fe)

Iron is an essential element in several biochemical and enzymatic processes. It is involved in the transport of oxygen to cells. However, at high concentration, it can increase the free radicals production, which is responsible for ageing and degenerative diseases (Jordao *et al.*, 2002).

2.5.2 Inorganic matters

2.5.2.1 Nitrate

High concentrations of nitrate cause methemoglobinemia in infants and could cause cancer. In the blood, nitrate convert hemoglobin to methemoglobin, which does not

carry oxygen to the body cells, which may lead to death from asphyxiation (Purushotham *et al.*, 2011).

2.5.2.2 Sulphate

Sulphate can be found in almost all natural water. The origin of most sulphate compounds is the oxidation of sulphite ores, the presence of shales, or the industrial wastes (Lenntech, 2015). Excessive sulphate concentration may lead to laxative effect and it affects the alimentary canal (Purushotham *et al.*, 2011). Animals are also sensitive to high levels of sulphate. In young animals, high levels of sulphate may be associated with severe, chronic diarrhea and in few instances death. As with humans, animals tend to become accustomed to sulphate over time (El-Hady, 2009).

2.5.2.3 Potassium

High potassium concentration may cause nervous and digestive disorders (Purushotham *et al.*, 2011), kidney and heart disease, coronary artery disease, hypertension, diabetes, adrenal insufficiency, pre-existing hyperkalaemia. Infants may also experience renal failure and immature kidney function (WHO, 2009).

2.5.2.4 Fluoride

High concentration of fluoride can cause dental and skeletal fluorosis such as mottling of teeth, deformation of ligaments and bending of spinal cord (Janardhana Raju *et al.*, 2009).

2.6 Corrective Actions to Environmental Problems Caused by Industrial Discharge

Several ways to solve the environmental problem caused by industrial discharge includes bioremediation, phytoremediation, and application of green chemistry among others. These corrective actions are discussed below with selective case studies.

2.6.1 Bioremediation

Conventional methods used by industries to cleanup pollutants usually involve physical treatment such as sedimentation and filtration, and chemical treatments such as flocculation, neutralization, and electro-dialysis. However, the treatment efficiency does not meet the regulation limits. Hence, further treatments are to be applied. Among all the technologies that have been investigated, bioremediation emerged to be the most desirable approach for cleaning up contaminant from industrial discharge (Shedbalkar and Jadhav, 2011).

Bioremediation can be defined as a technology that utilizes the metabolic potential of microorganisms to clean up contaminated environments (Wanatabe, 2001). Numerous studies have been carried out in search for appropriate and useful bioremediation agent, such as bacteria, yeast and fungi. Examples of bioremediation are bioaugmentation, biostimulation, land farming and composting (Boopathy, 2000). Many research have been carried out on bioremediation of contaminants such as petroleum and diesel oil (MacNaughton *et al.,* 1999; Watanabe, 2001), pesticide like DDT (Purnomo *et al.,* 2011), herbicide like Pendimethalin (Venkata Mohan *et al.*, 2007).

2.6.2 Phytoremediation

Phytoremediation is the use of plants to remove pollutants from contaminated environment. Wetland plants are important tools for heavy metals removal from aquatic

ecosystems and proved to be effective in metal pollution abatement because they chemically reduce metals by reacting with them (Rai 2011). *Azolla, Eichhornia, Typha, Phragmites, Lemna* and other aquatic macrophytes are important wetland plants for cleanup of heavy metals contaminated sites. These wetland plants absorb pollutants in their tissue and provide a surface and an environment for microorganisms to grow (Scholz, 2006; Rai, 2009; Ho *et al.,* 2014). Water hyacinth (*Eichhornia crassipes*) is commonly used in constructed wetlands because of its rapid growth rate and ability to take up lots of nutrients and contaminants (Rai, 2009). In addition, wetland plants can stimulate aerobic decomposition of organic matter and of nitrifying bacteria (Ho *et al.,* 2014). However, in a wetland system, microorganism has a better capability in organic matter degradation compared to wetland plant because as the organic matter binds with metals directly, they provide carbon and energy source for microbial metabolism (Stottmeister *et al*., 2003; Rai, 2011).

2.6.3 Green chemistry

Green chemistry is defined as the design of chemical products and processes that could reduce or eliminate the application and generation of hazardous substances (Kirchhoff, 2005; Tiwari *et al*., 2008; Rai, 2011). Green chemistry is one of the possible sustainable environmental managements. Three important areas in green chemistry are (i) Application of alternative synthetic pathways, (ii) Application of alternative reaction conditions and (iii) Design for safer chemical compounds that are less toxic or inherently safer with regards to accident potential (Garcıa-serna *et al.,* 2007; Rai, 2011). For example, research based on inexpensive materials, such as chitosan, zeolites and other adsorbents, which have high adsorption capacity and are economical have been conducted (Babel and Kurniawan, 2003). Rai (2011) reported the adsorption of Cu^{2+} and Cr^{3+} onto polyacrylonitrile composite. Similarly, Pehlivan and Arslan (2007)

reported the adsorption of lignite (brown young coals) to remove copper, lead and nickel from aqueous solutions at the low concentrations. Green chemistry was implemented to promote sustainability (Kirchhoff, 2005; Rai, 2011).

2.7 Heavy Metal Removal from Waste Water

2.7.1 Conventional methods

The methods used to remove metal ions from aqueous solutions include physical, chemical, and biological techniques. Many conventional methods can achieve this goal, such as chemical precipitation, ultra-filtration, ion exchange columns, electrochemical treatment, filtration membrane technologies, adsorption by activated carbon, and evaporation.

2.7.1.1 Chemical precipitation

The most widely used chemical method is chemical precipitation. This procedure is based on the formation of insoluble compounds of coagulants, which are then filtered out. Precipitation of metals is achieved by the addition of coagulants such as alum, lime, iron salts and other organic polymers. Various types of chemical precipitation include hydroxide precipitation which involve the use of calcium or sodium hydroxides to precipitate metals; carbonate precipitation uses sodium or calcium carbonates to precipitate metals and sulphide precipitation uses sulphides for metal precipitation. Although, chemical precipitation is one of the cheapest and the simplest methods, but the large amount of sludge containing toxic compounds produced during this process is the main disadvantage (Harris, 2008; Babak *et al.,* 2012).

2.7.1.2 Ion exchange

Ion exchange involves the use of resins which are selectively available for certain metal ions. The cation exchange resins are mostly synthetic polymers containing an active ion group. The cations are exchanged for H^+ or Na^+. The method of ion exchange is

effective, but quite expensive. Also, there are limitations on the use of ion exchange for inorganic effluent treatment which include; resin fouling, generally not effective at low pH, high concentrations of iron, manganese and aluminum and generally not effective for complex mixture of metals (Weaton and Bauman 2006; ITRC, 2015).

2.7.1.3 Ultrafiltration

This process involves ionic concentration by the use of selective membrane with a specific driving force. Ultrafiltration mainly includes reverse osmosis and electro dialysis. For reverse osmosis, pressure difference is employed to initiate the transport of solvent across a semi permeable membrane while electro dialysis relies on ion migration through selective permeable membranes in response to a current applied to electrodes. Micro-, nano- and ultrafiltration and reverse osmosis are the most commonly used physical methods in industries (Volesky, 2001). The disadvantages of this process are little efficacy at lower concentrations and the formation of secondary waste. This waste must be further processed or stored (Volesky, 2001; Ahluwalia and Goyal, 2007).

2.7.1.4 Adsorption

Adsorption is the physical adherence or bonding of ions and molecules onto the surface of another molecule i.e. onto two dimensional surfaces. In this cases, the material accumulated at the interface is the adsorbate and on the solid surface is adsorbent. Examples of these low cost adsorbents are peat, betonite, fly ash, china clay, maize cob, silica, active alumina, zeolite, metal oxides and so on. However, much attention in recent years is given to the use of activated carbon or activated alumina (Ahluwalia and Goyal, 2007; Rai, 2011; Jan *et al.*, 2014).

2.7.1.5 Solvent extractions

Solvent extraction involves an organic and an aqueous phase. The aqueous solution containing the metal or metals of interest is mixed with the appropriate organic solvent and the metal passes into the organic phase. In order to recover the extracted metal, the organic solvent is contacted with an aqueous solution whose composition is such that the metal is stripped from the organic phase and is re-extracted into the stripping solution (Ahluwalia and Goyal, 2007).

2.7.1.6 Cementation

Cementation is the displacement of a metal from solution by a metal higher in the electromotive series. It offers an attractive possibility for treating any wastewater containing reducible metallic ions. Cementation is especially suitable for small wastewater flow because a long contact time is required (Wang and Chen, 2009).

2.7.1.7 Chemical reduction

This process involves the reduction of toxic heavy metal into a less toxic one. Reduction of hexavalent chromium can be accomplished with electro-chemical units. The electrochemical chromium reduction process uses consumable iron electrodes and an electric current to generate ferrous ions that react with hexavalent chromium to give trivalent chromium. Another application of reduction process is the use of sodium borohydride, which has been considered effective for the removal of mercury, cadmium, lead, silver and gold (Wang and Chen, 2009).

2.7.1.8 Evaporators

This is commonly used in the electroplating industry. Evaporators are used chiefly to concentrate and recover valuable plating chemicals. Recovery is accomplished by boiling sufficient water from the collected rinse stream to allow the concentrate to be

returned to the plating bath. Both capital and operational costs for evaporative recovery systems are high. Chemical and water reuse values must offset these costs for evaporative recovery to become economically feasible (Volesky, 2001; Ahluwalia and Goyal, 2007).

The conventional methods have significant disadvantages which include incomplete metal removal, high capital costs, high reagent and/or energy requirements, and generation of toxic sludge or other waste products that require disposal (Göksungur *et al.*, 2005). These disadvantages, together with the need for more economical and effective methods for the recovery of metals from waste waters, have resulted in the development of alternative separation technologies.

2.7.2 Biological methods of heavy metal removal

In recent years, research attention has been focused on biological methods which have emerged as an effective alternative option in place of conventional methods of metal sequestration. In biological methods, biomass derived from various biological sources is utilized and their property and potentials of interaction with targeted pollutant have been harnessed for biotreatment. Fundamental to these biotreatment processes are the activities of living organisms, upon which transformation and detoxification of heavy metal pollutants depends (Gadd and White, 1993; Volesky and Holan, 1995; Gadd, 2010).

The biological treatment can be classified into two principle categories namely active and passive processes. The active or metabolically dependent process (bioprecipitation, biomineralization and bioaccumulation) involves the use living and active biological materials. The passive or non-metabolic dependent process (biosorption) involves the use of dead or inactive biomaterials (Atkinson *et al.*, 1998; Malik, 2004). Many

biological materials from plants and microbes can bind to all type of heavy metals but only those with sufficiently high binding capacity and selectivity for heavy metals are suitable for use in bioremediation processes (Gomez *et al.,* 2004; Gadd, 2010). A large number of microorganisms belonging to various groups (cyanobacteria, bacteria, yeast and fungi) respond to heavy metals in different ways depending on the nature of the microorganisms and on the concentration of the heavy metal in the environment (Payne, 2000). Among these microorganisms, fungal biomass offers the advantage of having a high percentage of cell wall material which shows excellent metal-binding properties, easy cultivation at large scale as it has short multiplication time and easy availability of fungal biomass as industrial waste product.

Biological method depends not only on the chemical composition of the cell or its components such as the cell wall, but also on external physicochemical factors and the solution chemistry of the metal. A combination of mechanisms involved in bioaccumulation and biosorption include: Particulate ingestion or entrapment by flagellae or extracellular filaments, active transport of ions, ion exchange, complexation, adsorption, inorganic precipitation, co-ordination and chelation. While the first two mechanisms are associated with living cells, the latter mechanisms have been reported for living and dead microorganisms, as well as cellular debris (Payne, 2000). The sequestered metals may be found in the extracellular polysaccharides, mucilage, capsule or cytoplasmic granules, depending on the microbial species and the mechanism of metal deposition within the cell (Harris and Ramelow, 1990; Payne, 2000).

Bioaccumulation and biosorption are superior to precipitation in terms of ability to adjust to changes in pH and heavy metal concentrations, and superior to ion exchange in terms of sensitivity to the presence of suspended solids, organics and the presence of

other heavy metals (Payne, 2000). Studies have been reported on the use of microorganisms in the removal of heavy metals (Jonathan *et al*., 2008; Radic *et al.*, 2010). Many studies have also been carried out in the use of fungi in the removal of heavy metals from waste water (Vianna *et al*., 2000; Tan and Cheng, 2003). The most popular fungal genera used include *Aspergillus*, *Penicillium*, *Rhizopus*, *Trichoderma*, and *Fusarium.* Some of these fungi and their heavy metal uptake are described in Table 2.2.

2.8 Advantages of Biological Method over Conventional Method of Metal Removal

Overall, the points below give the potential advantages of biological methods over conventional methods of metal removal (Wilde and Benemann, 1993; Payne, 2000). These include:

1. Greatly improves the recovery of bound heavy metals from biomass
2. Greatly reduces the production of large volume of hazardous waste
3. High affinity in reducing residual metals to below 1 part per billion (ppb) in many cases
4. Highly selective in terms of removal and recovery of specific heavy metals
5. There is less need for additional expensive process reagents which typically cause disposal and space problems.
6. It has low capital investment and low operating costs.
7. It has the ability to handle multiple heavy metals and mixed waste.
8. It requires the use of naturally abundant, renewable biomaterials that can be produced cheaply.

Table 2.2: Metal Uptake by Fungal Biomass

Fungi	Metal ions	Uptake capacity (mg/g)	References
Penicillium spp.	Pb	6.0	Say *et al.* (2003)
Aspergillus niger		34.4	Dursun *et al.* (2003)
Rhizopus nigricans		403.2	Kogej and Pavko (2001)
Phanerochaete chrysosporium		419.4	
Trichoderma sp.		60	
Fucus vesiculosus.	Ni	2.85	Bakkaloglu *et al*. (1998)
Saccharomyces cerevisiae		1.47	
Aspergillus nodosum		1.11	
Aspergillus oryzae	Cd	5	Vianna *et al*. (2000)
Penicillium spp.		3	Deng and Ting (2005)
Aspergillus niger		1.31	Kapoor *et al.* (1999)
Penicillium chrysogenum	Cr	18.6	Tan and Cheng (2003)
Penicillium chrysogenum	Zn	19.2	Bakkaloglu *et al.* (1998)
Fusarium vesiculosus		17.3	
Saccharomyces cerevisiae		3.45	
Penicillium spp.	Cu	3	Kapoor and Viraraghavan
Aspergillus niger		15.6	(1997)
Aspergillus awamori		43	Gulati *et al*. (1997)
Aspergillus oryzae			

2.9 Mechanisms of Microbial Accumulation

The complex structure of microorganisms gives an insight on different ways by which metals can be taken up by the microbial cell. There are various metal accumulation mechanisms classified according to various criteria. Based on cell metabolism dependence, metal accumulation mechanisms can be divided into: Metabolic dependent and Non -metabolic dependent. Based on the location of the cell where the metal is removed from solution, metal accumulation can be classified as extra cellular accumulation, cell surface sorption/precipitation and intracellular accumulation (Gadd and Sayer, 2000; Payne, 2000).

2.9.1 Metabolic dependent mechanism

Metabolic-dependent mechanism is a phase of metal accumulation that occurs in living cells which may be accompanied by toxic symptoms. Transportation of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of accumulation may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal. In some cases, intracellular uptake is due to increased membrane permeability arising from toxic interactions. When metal ions gain access into cells, they may be preferential within specific organelles and or bound to proteins such as metallothionein. The accumulation of some metals such uranium, thorium and lead by microbial biomass is surface based with little or no intracellular uptake except by diffusion (Payne, 2000).

2.9.2 Non-metabolic dependent mechanism

Non-metabolism dependent mechanism is an initial, rapid phase which occurs in dead cells. It involves metal uptake by physicochemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cell's metabolism. Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids have abundant metal binding groups such as carboxyl, sulphate, phosphate and amino groups. This type of mechanism i.e., non-metabolism dependent is relatively rapid and can be reversible (Gadd and Sayer, 2000).

Metabolism and non-metabolism dependent mechanisms of metal uptake in growing cultures may be affected by changes in medium composition and excretion of metabolites that can act as metal chelators. Thus in a given microbial system, mechanisms of uptake may operate simultaneously or sequentially (Veglio and Beolchini, 1997; Payne, 2000).

2.10 Interactions and Transformation of Metal and Metalloids by Fungi

The processes by which microorganisms interact with toxic metals are very diverse. Metal sequestration by microbial biomass follows complex mechanisms, mainly ion exchange, chelation, adsorption by physical forces and ion entrapment in inter and intra fibrillar capillaries and spaces of the structural polysaccharide networks of cell wall and membranes. Cell walls of biomass consist of polysaccharides, proteins and lipids, which offer various functional groups like carboxylate, hydroxyl, sulphate, phosphate etc. for the binding with metal ions (Zafar *et al.*, 2007). The cell walls of the biomass in general have a negative charge, thus high affinity towards the cationic species of the metal ions.

Fungi play an important role in carbon mineralization and biogeochemical cycles in the terrestrial and aquatic environment. They are important decomposers and plant symbionts/mycorrhizas (Gadd and Sayer, 2000). The branching filamentous explorative growth habit and high surface area to mass ratio of fungi ensures that their interactions with heavy metals are an integral part of environmental cycling process (Gadd and Sayer, 2000).

The various ways in which metals and their derivatives can interact with metals depends on the type of metal, the organism and the environment and as well depends on influence of environmental factors like pH and temperature. Metals, whether soluble or insoluble can interact with fungi through various ways (Figure 2.1): (i) metal solubilization (ii) metal mobilization (iii) metal immobilization (iv) particulate adsorption and entrapment by mycelia network.

Metal solubilization involves metabolite excretion of organic acid and hydrogen ions, siderophores, methylation, heterotrophic leaching and biodegradation of organometalloids. Soluble metals interact with fungi via biosorption and bioaccumulation, transport, intracellular sequestration and compartmentation, redox reactions, precipitation and crystallization Insoluble metals interact with fungi through particulate adsorption, entrapment by polysaccharides and mycelia network (Wang and Chen, 2009).

Metal immobilization occurs by crystallization and reduction. Environmental factors apart from influencing nutrient, salinity, toxic metals and other pollutants on fungal growth, metabolism and morphorgenesis, they also direct equilibrium between soluble and insoluble metals towards mobilization and immobilization.Fungi on the other hand,

influence the environment through alteration in pH, oxygen, carbondioxide, redox potential, depletion of nutrients, enzymes and metabolite secretion.

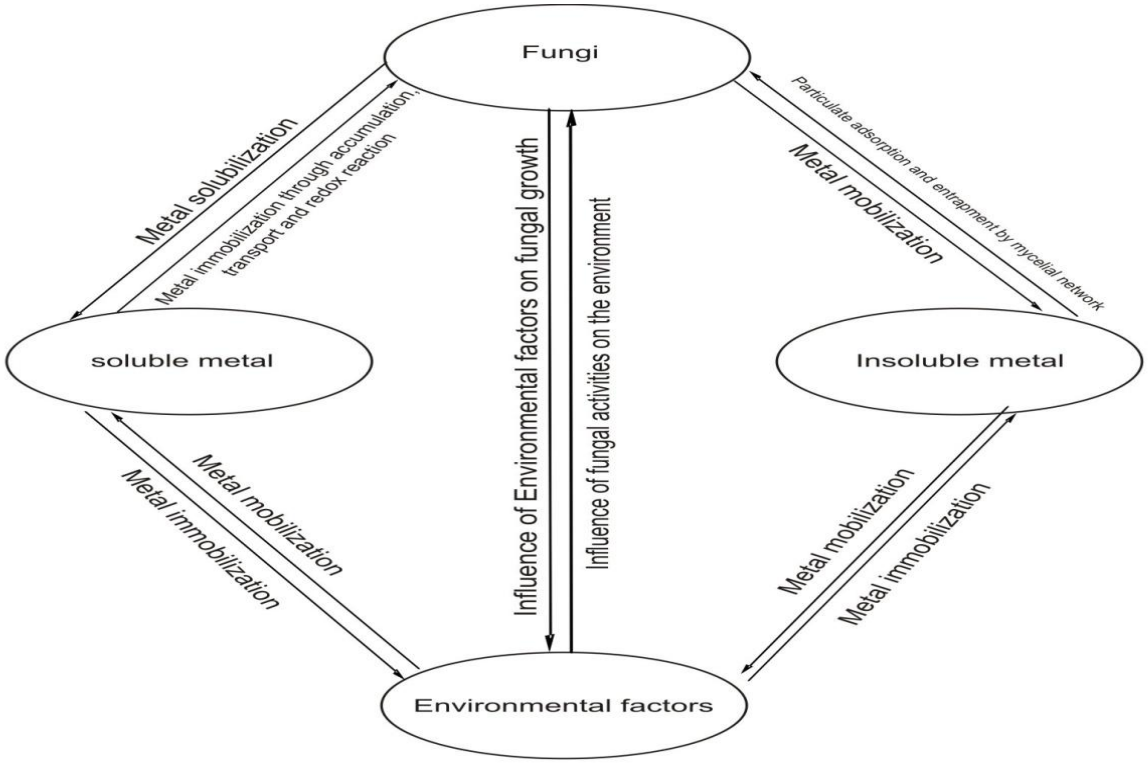


Figure 2.1: Interactions and Biogeochemical Transformations of Metals by Fungi

Source: Gadd and Sayer (2000)

2.11 Factors affecting Heavy Metal Accumulation

Bioaccumulation process is influenced by several factors that may have a significant influence on the sorption capacity of the sorbent or sorption. Metal accumulation is mainly influenced by pH, concentration and type of biomass, the presence of other metals, temperature and initial concentration of metal (Ahalya *et al.*, 2003).

2.11.1 pH

pH is the most important parameter in metal accumulation. It affects the solubility and solution chemistry of metal, the activity of the functional groups of cell walls of the biomass and competitiveness of the metal ions (Ahalya *et al.*, 2003). The availability of free binding sites varies depending on the pH. At lower pH, all binding sites are protonated which prevents the access of positively charged metal ions and complete desorption of linked metal ions (Reya *et al.*, 2012). On the other, extreme pH values may damage the structure of the biosorbent, cell deformation and reduction in sorption capacity. Higher pH significantly reduces the solubility of metals, metal hydroxides formation, which collide and thus impede biosorption since most metals are found as the optimum pH range 3–6 (Kratochvil and Volesky,1998; Ahalya *et al.*, 2003; Volesky and Naja, 2007).

2.11.2 Concentration and type of biomass

A large number of different types of biomass have been studied in terms of their sorption properties. Algae, bacterial biomass, biomass of fungi and plants. Metal accumulation does not only depends on the microbial species but also dependent on growth conditions (culture medium), physiological condition and age of biomass. High biomass concentration is very effective at absorbing various metals while lower biomass concentration leads to a higher intake of specific metal and interference between the

binding sites (Ahalya *et al.*, 2003; Naja *et al.*, 2005). Fourest *et al.* (1994) invalidated this hypothesis attributing the responsibility of the specific uptake decrease due to metal concentration shortage in solution. Hence, this factor needs to be considered in any application of microbial biomass as biosorbent (Das *et al.*, 2008).

2.11.3 Temperature

Temperature affects the stability of the metal in solution, the configuration of the cell wall or stability of complex cells with bound metal. In general, temperature has less influence than other factors, especially if it is between 20°C and 35 °C. Biosorption of some metals (uranium and copper) may take place without restrictions in a wide temperature range (Ahalya *et al.*, 2003; Ozturk *et al.*, 2004; Volesky and Naja, 2007).

2.11.4 Presence of other metals

The rate of inhibition of metal accumulation depends on the strength of the individual metals bounded to the biomass. Generally, light metals (alkali and alkaline earth metals) bind less to biomass than heavy metals or radioactive elements. Thus, the presence of very light metal does not affect sorption of heavy metals. Among heavy metals is weakly bound zinc, which is more influenced by other metals (Volesky and Naja, 2007; Reya *et al.*, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

Kaduna Refinery and Petrochemical Company (KRPC) and Romi River both located in Chikun local government area of Kaduna state Nigeria were used for this study. The refinery occupies an area of 2.9 square kilometers and is located on an undulating land about 700meters above sea level (Lekwot *et al.,* 2012). This elevation is equivalent to about 17meter higher relative to Romi River (Lekwot *et al.,* 2012).

Romi River is one of the tributaries of River Kaduna, located in the southern part of Kaduna metropolis and follows a course of about 16.4 km. The Romi River receives effluent from the petrochemicals and refinery waste treatment facilities via a tributary called the railway bridge stream which flows about 1km to Romi River. The general direction of flow of the river is South west-north west of Kaduna town. The river runs through Angwan Romi village where there are human settlement, agriculture, industries and solid waste disposal site. The river provides breeding ground for aquatic animals. It is also a source of water for drinking and recreational uses for public and as a repository for industrial and domestic wastes.

3.2 Sampling Sites

3.2.1 Effluent Sample Site

Effluent samples were collected from the effluent sites (A, B, C) of the Kaduna Refinery and Petrochemical Company (KRPC). Sites A and B are located in the waste water treatment plant while site C is located outside the company.

I Site A is called the untreated waste water channel which retains the untreated effluent for some time before flowing into point B for treatment.

II Site B which is about 100m from point A, is called the waste oil retention pond where the untreated waste water from point A flows into. Here caustic soda is added during treatment to neutralize the acid. Booms and skimmers are placed in this point to trap oils from the waste water.

III Site C located outside the refinery, is called the discharge point which has a distance of about 600m from point B. Point C discharges the refinery effluent directly into the railway bridge stream; a tributary, which flows directly into Romi River which is a tributary of River Kaduna.

3.2.2 Romi River

Two locations; 100 meters each, of upstream (site D) and downstream (site E) of the Romi River from the discharge point were used for sampling following the method described by Lekwot *et al*. (2012). The reasons for selecting these locations were to determine the physicochemical properties of the river as affected by discharged effluent and to isolate fungi in these locations.

The map of the study site and pictorial representation of the study sites are presented in Figure 3.1.and Plate 3.1.

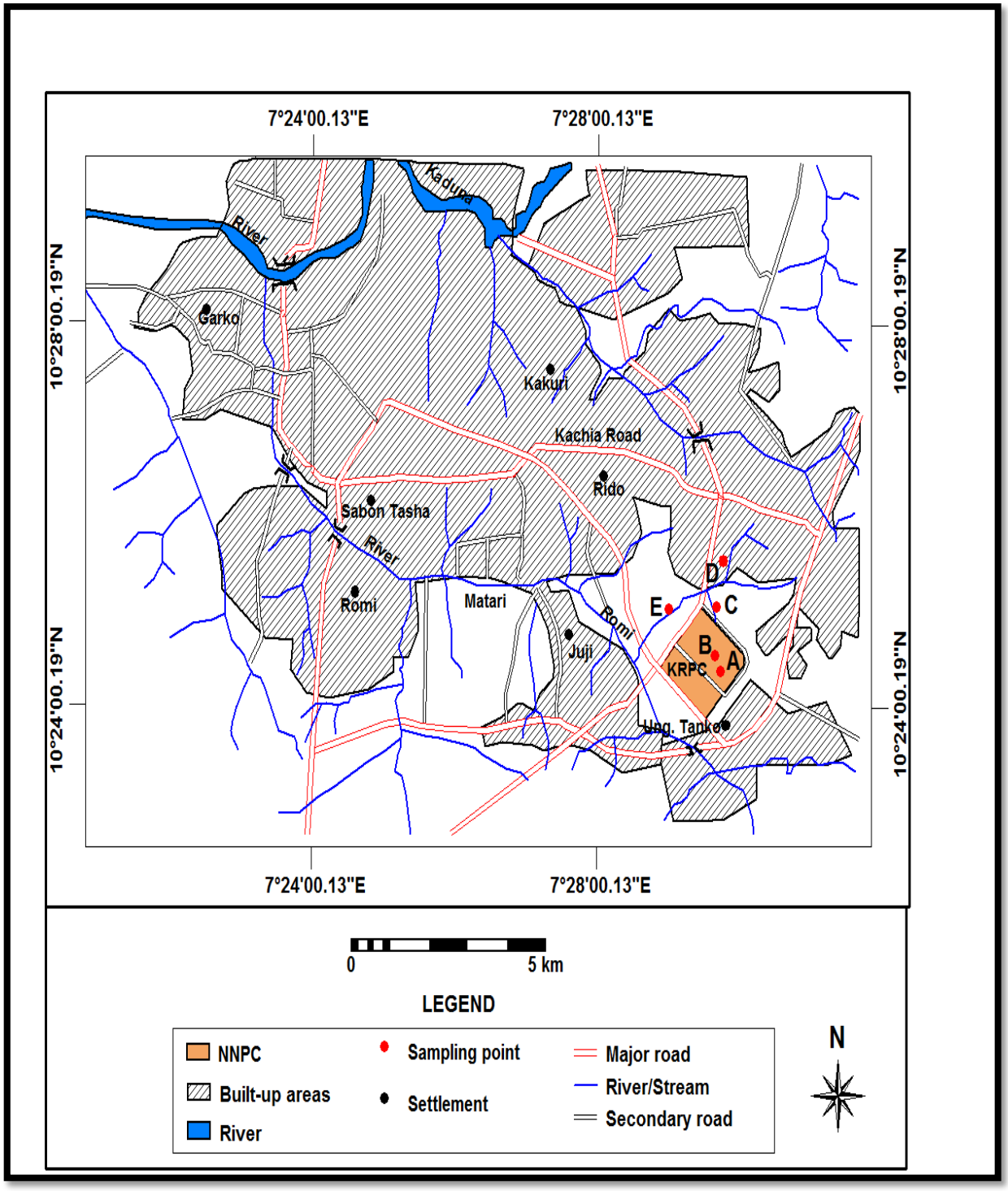


Figure 3.1: Part of Kaduna South Showing the Study Site and the Various Sampling Sites

Source: Derived from Google map, 2014 and digitized in GIS Lab. Geography department, A.B.U Zaria



Plate 3.1a: Untreated Waste Water Channel (Site A)



Plate3.1b: Waste Oil Retention Pond (Site B)



Plate 3.1d: Discharge Point (Site C)



Plate 3.1d: Upstream (Site D) and Downstream (Site E) of Romi River

3.3 Collection of Refinery Effluent and Water Samples from Romi River

Effluent from KRPC and water samples from Romi river were collected in sterile sample bottles from five sites namely; untreated waste water channel (site A), waste oil retention pond (site B), Discharge point (site C), Upstream of Romi river (site D) and downstream (site E) of Romi river. The samples were collected in duplicates by lowering the bottles into the well mixed section of the site, 30cm deep and allowed to overflow before withdrawing. All sample bottles were properly labeled to indicate the sample code, collection point, date and sampling time. After collection, the bodies of the bottles were rinsed thoroughly with sterile distilled water before transporting them in ice box to the laboratory for fungal isolation, physicochemical and heavy metal analysis.

3.4 Physicochemical Analysis of the Refinery Effluent and Romi River

This was carried out in accordance with the methods of Thipeswamy *et al*. (2012). Physicochemical analysis were carried out to know the natural conditions of the samples, extent of pollution of the receiving water body, physical and chemical conditions under which the potential organisms to be isolated exist. The physicochemical parameters analyzed include; pH, temperature, electrical conductivity, dissolved oxygen (DO), biological oxygen demand (BOD), sulphate, nitrate and phosphate while the heavy metals; lead (Pb), nickel (Ni), and Cadmium (Cd) were determined using fast sequential atomic absorption spectrophotometer (model AA240FS, England).

3.4.1 pH, temperature, electrical conductivity and total dissolved solids

The pH, temperature, electrical conductivity and total dissolved solids of the effluent and water samples were determined at the point of collection using the HANNA combo

tester (H198130, Denver, USA), a water proof tester that offers high accuracy of pH, EC, TDS and temperature in a single test). Briefly, following the manufacturer's instructions, the electrodes connected to each meter were submerged in a clean bucket containing the sample. The values read for each parameter by the tester was observed and recorded.

3.4.2 Dissolved oxygen and biological oxygen demand

The Hanna instrument (HANNA 3200, Denver, USA) was used to determine the dissolved oxygen (DO) and biological oxygen demand (BOD). The instrument was inserted into the sample to determine the DO at the point of collection while the BOD was obtained after the sample was collected, transferred to a BOD bottle and allow to stand for 5days at room temperature (28±30^{o}C). The BOD was determined by inserting the instrument into the incubated sample and then subtracted the initial DO from the final value to obtain the BOD_5 (Radojavic and Bashkin, 1999).

3.4.3 Nitrate

This was carried out by the method described by APHA (1999). The sample was neutralized at pH 7.0.One hundred millilitres (100 ml) of the sample was placed in a beaker and allowed to evaporate to dryness on a water bath. The residue was dissolved using a glass rod with 2ml disulphuric acid reagent and then transferred to Nessler's tube. Six millilitres (6 ml) of ammonium hydroxide (NH_4OH) and EDTA was added drop wise till the residue dissolves. A blank was also prepared in the same way using distilled water instead of the sample. The colour development was read at 410nm with a light path of 1cm.The nitrate concentration was calculated using the formula:

$$\text{mg/litre of nitrate, } N = \frac{\text{mg nitrate N} \times 1000}{\text{ml sample taken}}$$

The nitrogen concentration was estimated by comparing the reading with a standard curve previously prepared.

3.4.4 Sulphate

One hundred millilitres (100ml) of the sample was dispensed in a 250 Erlenmeyer flask, 5 ml conditioning reagent was added and mixed using a magnetic stirrer. A spoonful of barium chloride crystals was added while the solution was still being stirred. The turbidity was measured at 30seconds after stirring at interval of 4 minutes using turbidity meter (Hach 2100N, USA). A blank was run with no barium chloride added. The sulphate concentration was calculated using the formula:

$$\text{mg/l of sulphate } (SO_4) = \frac{\text{mg } SO_4 \times 1000}{\text{ml sample taken}}$$

The sample was estimated by comparing the turbidity reading with a standard curve previously prepared.

3.4.5 Phosphate

3.4.5.1 Preliminary sample treatment

One drop (0.05ml) phenolphthalein indicator was added to 100ml sample. Strong acid solution (hydrochloric acid) was added drop wise when the colour turned pink, to discharge the colour.

3.4.5.2 Colour development

One hundred millilitres (100 ml) of treated samples was poured into a flask and 4.0ml of molybdate reagent was added and mixed thoroughly. Ten drops (0.5ml) stannous chloride reagent was added. After 10 minutes, the colour was measured photometrically at 690nm and compared with the standard curve. Distilled water was used as blank. Calculation was carried out using the formula:

$$\text{mg/ l of phosphate } PO_4 = \frac{\text{mg } PO_4 \times 1000}{\text{ml sample}}$$

3.4.6 Oil and grease

The Partition – Gravimetric method was used for the analysis of oil and grease. 50 millilitres (50 ml) of each sample was acidified to pH 2.0 using 5ml of hydrochloric acid, shaken vigorously for three minutes. The sample was transferred into a separating funnel held by a retort stand, and then 15ml of hexane was used to carefully rinse the beaker before pouring the sample into the funnel which separated the content into layers of oil, grease and water. The water being of higher density was collected below leaving oil and solvent at the surface. Twenty grams of anhydrous sodium sulphate was added to the filter cone before the emulsified solvent was collected. Extraction was done twice using 15ml of the organic solvent in each process. An additional 20 ml of solvent was used to wash the filter paper and evaporation was carried out using water bath until all the solvent evaporate. The flask was transferred to a desiccator before weighing. The oil and grease content was determined as follows:

$$\frac{A - B \times 1000\text{ml of oil and grease}}{\text{ml sample oil + grease (mg/l)}}$$

Where, A and B are the initial and final weight of the flask and its contents.

3.4.7 Determination of Heavy Metal in Effluent and Water Samples

The concentrations of heavy metals (lead, nickel and cadmium) in the samples were analyzed using the fast sequential atomic absorption spectrophotometer (Model AA240S, Varian technologies, USA) following the modified method of APHA (1995). Briefly, the instrument's settings and operational conditions were done in accordance with manufacturer's specifications by calibrating with analytical grade metal standard stock solution.

Five millilitres (5ml) of each sample was digested in a beaker by adding 37.5ml of nitric acid and 12.5ml of hydrochloric acid, heated to almost dryness and topped up to 50ml with distilled water. The digested samples were filtered to remove any insoluble materials that could clog the atomizer. The filtrate was then analyzed for heavy metals using the AAS.

3.5 Microbiological Analysis

3.5.1 Isolation of fungi from the samples collected

The microbiological analysis of the samples was carried out the same day of sample collection to avoid microbial deterioration of the samples. Briefly, the samples were kept to stand at room temperature (28±30^{o}C) on a sterile laboratory work bench. 9ml of each sample in duplicates were aseptically dispensed in sterile centrifuge tubes and centrifuged at a speed of 250rpm for 10minutes to concentrate the samples. After decanting the supernatant, 0.1ml of the residue of each sample was spread-plated on different sterile solidified laboratory prepared media (potato agar, Potato carrot agar, potato agar with 7.5% NaCl) in duplicates containing 50µg/L of chloramphenicol using sterile bent glass rod. The plates were incubated at room temperature in a disinfected dark cupboard for 7days. Distinct colonies from incubated culture plates were assigned isolation codes; sub cultured and transferred aseptically into potato dextrose agar slants for identification and further laboratory analysis. More than one mycological media was used to enhance the chances of securing more fungal isolates. The choice of the media used was for isolating fungi from environmental samples.

3.5.2 Identification and characterization of the fungal Isolates

Macromorphological characteristics of the fungal isolates, such as colour, texture, colour of the reverse side of the fungal isolates were observed and recorded. For the micromorphological characteristics, small portion of the growing region was mounted

on clean grease free slide with a drop of lacto phenol cotton blue, covered with a cover slip which was examined by microscopy using ×40 objective lens. Characteristics of the sexual reproductive structures, presence or absence of septation, presence of foot cells and chlamydospores were observed and recorded. Each fungal isolate was identified using appropriate taxonomic guide (Barnett and Hunter, 1999; Klich, 2002; Larone, 2002; Nagamani *et al*; 2006; Hakeem and Bhatnagar, 2010; Thipeswamy *et al*; 2012). The Isolated pure cultures were maintained in agar slants and stored in a refrigerator.

3.6 Preparation and Standardization of Fungal Spore Inoculum

This was carried out following the modified methods of Belli *et al.* (2005)*;* Bekada *et al.* (2008) and Bekker *et al.* (2009). Briefly, the fungal isolates were grown on potato dextrose agar slant for 7days at 28±30^{o}C to obtain heavily sporulated cultures. The spores were scraped gently using a sterile inoculation needle under sterile aseptic conditions. Spores suspension of the isolates were obtained by dispensing 15ml of sterile distilled water containing 0.005% Tween 80 (Probus, Barcelona, Spain) into the agar slant and shaken properly by vortexing for 15minutes, to wash off the spores. The spore suspensions were diluted with sterile distilled water to obtain a concentration of 6×10^{8} spores/ml. The spores were enumerated by direct counting, using Neubauer chamber (hemocytmeter). Following the method of Joseph *et al.* (1998), these standardized spore suspensions of each isolate was stored for up to 5days at 4^{o}C in the refrigerator and used when required.

3.7 Determination of Lead, Nickel and Cadmium Tolerance by the Fungal Isolates

3.7.1 Preparation of stock solutions of heavy metal ions

Stock solutions of lead nitrate ($Pb(NO_3)_2$), nickel sulphate ($NiSO_4$) and cadmium sulphate were prepared by dissolving 0.1g of each analytical grade salts in separate

Erlenmeyer flasks containing 500ml of distilled water. The flasks were warmed over hot plate with vigorous shaking to obtain a clear solution of to obtain 200µg/ml concentrations which was sterilized by autoclaving at 121^{o}C for 15 min. The solutions were stored in a refrigerator at 4^{o}C for later use.

3.7.2 Bioassay procedure for heavy metal resistance

The fungal isolates were assayed for their capacity to resist and grow in the presence of 5, 10 and 15 µg/ml of the test heavy metal ions invitro. The yields of biomass in liquid shake cultures were used as index of resistance and growth in the presence of experimental concentrations of the heavy metals (Bennet *et al.,* 2002). Each test isolate was inoculated in duplicate conical flasks containing 100ml of freshly prepared potato dextrose broth supplemented with 5, 10 and 15 µg/ml each of Pb, Ni, and Cd. Appropriate controls without heavy metals were used for comparative evaluation. The inoculated flasks were incubated at room temperature (28±30^{o}C) aerobically on a rotatory shaker for 7 days.

The mycelial mats produced were harvested by filtering the cultures through preweighed Whatman filter paper (No 1). The filter paper bearing the mycelial mats were dried in an oven at 70^{o}C for 48hours and re weighed. The yield of dry mycelial biomass was obtained by subtracting the weight of the filter paper alone from the weight of the filter paper and the mycelial biomass (Malik and Jaiswal, 2000; Bennet *et al.,* 2002).

Tolerance was measured as the growth of the fungi in the presence of heavy metals divided by growth in the same period in the absence of metal. Isolates which showed significant growth and tolerance to high concentrations of the heavy metals were ranked and used for subsequent steps (Burgess and Almeida, 2012; Kumar *et al.,* 2012).

3.8 Determination of the Optimum pH and Temperature Conditions of the Isolates

Temperature and pH are some of the key factors in the growth, behavior and development of fungal mycelium. Understanding the influence of these factors is relevant for biotechnology application of bioremediation as well as morphogenesis of filamentous fungi in general (Bekada *et al.,* 2008). They strongly influence carbon availability, metal solubility and nutrient availability as well as play important roles in enhancing biomass production, rate of nutrient absorption and efficient bioaccumulation process (Hamzah *et al.,* 2012).

3.8.1 pH optimization

The effect of pH of the medium on the growth of heavy metal resistant fungal isolates was investigated at varying pH of 4.0, 5.0, 6.0, and 7.0, by adjusting the pH of potato dextrose broth (PDB), using 0.1N hydrochloric acid and 0.1N sodium hydroxide before sterilization (Bekada *et al.,* 2008; Bekker *et al.,* 2009). 100ml Erlenmeyer's flask containing 50ml of pH-adjusted PDB was inoculated with 6×10^8 spores/ml of each of the fungal isolates After incubation at room temperature (28 ± 30^oC) for 7 days in a sterile dark cupboard, the content of each flask was aseptically filtered through a pre-weighed sterile Whatman filter paper, rinsed with distilled water, dried in hot air oven at 70^oC for 18hours and weighed.

3.8.2 Temperature optimization

The fungal isolates were inoculated into 100 ml conical flasks containing 50ml of PDB. The flasks were incubated at varying temperatures (25, 30, 35°C) for 7 days. The content of each flask was filtered, dried and weighed Hamzah *et al.*, 2012)

3.9 Determination of the Potential of the Resistant Isolates to Bioaccumulate and Remove Pb, Ni and Cd from Refinery Effluent

The heavy metal resistant fungal isolates were evaluated for uptake of lead, nickel and cadmium in potato dextrose broth containing the treated effluent sample sterilized at standard condition of 15lbs/psi for 15minutes at 121^{o}C. This was carried out following a slightly modified procedures of Jaeckele *et al.* (2005), Joshi (2011), Dwivedi *et al*. (2012) and Usman *et al.* (2012). The broth and effluent were added in the ratio of 4:1 (120ml of Potato dextrose broth and 30ml of the sterilized effluent sample) in 250ml Erlenmeyer flask for each tolerant fungal isolate.

Prior to the batch procedure, the initial heavy metal contents in the test fungi were determined by digesting and analyzing the fungal mycelium using AAS. Briefly, dried fungal mycelium of each test isolate was digested by adding 37.5ml of nitric acid and 12.5ml of hydrochloric acid, heated to almost dryness and topped up to 50ml with distilled water. The digested samples were filtered and the filtrate of each fungal isolate was then analyzed for heavy metals using the AAS.

The batch procedure for the bioaccumulation and removal of heavy metals was as follows:

Fourteen sets of 250ml flask were set for the work. The flasks were arranged in two sets. For the first set, the following were carried out:

1. 150ml of the broth was added to the first flask and left un-inoculated (control A)

2. 120ml of the broth was added into each of the remaining six flasks
3. 30ml of unsterilized effluent sample added into the second flask containing 120ml of the broth medium (control B)
4. 30ml of sterilized effluent sample was added into each of the remaining five flasks
5. The third flask (now containing 120ml of broth and 30ml of sterilized effluent sample) was not inoculated with fungal isolate (control C)
6. Each of the resistant fungal isolates was inoculated (6×10^8 spores/ml) into the remaining four flasks of four sets respectively.
7. The other seven flasks (which is the duplicate of the above steps) were arranged in the order described above. The flasks were incubated at room temperature (28 ± 30^oC) on a rotatory shaker at 150rpm for 14days.

After incubation, the content of each flask was aseptically filtered on a pre-weighed sterile Whatman filter paper (No.1) to separate mycelial mats from the culture filtrate the harvested mycelial mat was rinsed with distilled water. The filtrate was analyzed for lead, nickel and cadmium using atomic absorption spectrophotometer (AA240FS). The filter paper along with the mycelial mat were dried in hot air oven at 70^oC for 18hours and weighed thereafter, using analytical weighing balance. Difference between the weight of the filter paper bearing the mycelial mat and weight of the pre-weighed filter paper represent the fungal biomass, expressed in terms of dry weight of mycelial mat (mg/flask). The heavy metal uptake was calculated using the formula below, in accordance with the methods Viraraghavan and Yan (2000); Burgess and Almeida (2010); and Joshi *et al.* (2012).

$$Q = V\frac{(Ci-Cf)}{W}\times 1000$$

Where **Q** is the concentration of the heavy metal uptake accumulated by the fungal biomass (mg/g), **Ci** and **Cf** are the concentrations of the metal ions on the fungi before and after accumulation respectively. **V** is the total working volume (L) and **W** is the dry weight of the fungal biomass.

The heavy metal removal from the effluent was calculated in percentage using the equation:

$$\text{Heavy metal removal} = \frac{(X-Y)}{X} \times 100\%$$

Where, **X** and **Y** are the initial and final heavy metal concentrations in the effluent respectively, before and after introduction of the test isolates.

3.10 Statistical Analysis of Data

Statistical analyses of the data obtained from this study were carried out. Means were compared using one way Analysis of Variance (ANOVA) and Duncan multiple range test of post Hoc test ($p<0.05$). The results were also presented in Tables, Plates and Figures.

CHAPTER FOUR

RESULTS

4.1 Physicochemical Properties of the Refinery Effluent and Water Samples from Romi River

Table 4.1 shows the physicochemical properties of the five sampling sites (A, B, C, D and E). The data indicated that there were significant differences in pH of the samples collected from the different sampling sites. These differences in order of increasing pH along the sites were A<B<C=D<E. However, the acidic pH of the untreated waste water channel (site A) with the least mean±SE of 4.17±0.07 was below the permissible limit (6.0-9.0) of Federal Ministry of Environment (FEMNV), Nigeria. Temperature of all the sampling sites were within the permissible limit (40^{o}C) of FEMNV. However, temperature at the downstream was the lowest with a value of 29.53±0.07 ^{o}C and highest at the discharge point with a value of 36.5±0.29 ^{o}C. The Electrical conductivity (EC) of the sites were within the permissible limit (2µS/cm) of FEMNV. However significant differences observed in EC of the sampling sites were in this increasing order, B>C>E>A>D. Total dissolved solids (TDS); 743.00±4.04mg/l, 732.33±2.03mg/l, and 678.00±11.14mg/l at sampling sites B, C and E respectively were above the permissible limit (500mg/l) recommended by FMENV. However, the values of dissolved oxygen, biological oxygen demand, nitrate, sulphate, phosphate and oil and grease, clearly revealed that they were within the permissible limits of FMENV, Nigeria. There was significant difference (P<0.05) between the means of physicochemical parameters and the sampling sites.

Table 4.1: Physicochemical Properties of the Refinery Effluent and Romi River

Physicochemical parameters	Sampling points					F value	P value	Permissible limit
	A	B	C	D	E			FMENV
pH	4.17±0.07^{d}	6.17±0.09^{c}	6.83±0.07^{ab}	6.67±0.09^{b}	6.97±0.21^{a}	171.46	0.00	6.0-9.0
Temperature (°C)	31.03±0.08^{c}	35.13±0.67^{b}	36.5±0.29^{a}	29.53±0.07^{d}	35.2±0.06^{b}	184.33	0.00	40
Electrical conductivity (µS/cm)	0.88±0.01^{d}	1.65±0.01^{a}	1.54±0.03^{b}	0.18±0.01^{e}	1.39±0.01^{c}	1466.00	0.00	2
Total dissolved solids (mg/l)	405.67±8.29^{c}	743.00±4.04^{a}	732.33±2.03^{a}	81.33±3.53^{d}	678.00±11.14^{c}	1800.00	0.00	500
Biological oxygen demand (mg/l)	3.10±0.06^{b}	0.87±0.02^{d}	3.18±0.02^{b}	2.64±0.04^{c}	3.70±0.05^{a}	728.91	0.00	10
Dissolved oxygen (mg/l)	2.93±0.09^{b}	0.68±0.01^{d}	1.21±0.01^{c}	1.32±0.03^{c}	3.20±0.01^{a}	683.18	0.00	10
Nitrate (mg/l)	22.60±0.31^{a}	24.33±0.88^{a}	20.33±0.67^{b}	18.17±0.44^{c}	18.73±0.73^{bc}	20.59	0.00	
Sulphate (mg/l)	32.17±0.60^{a}	31.40±0.03^{b}	27.80±0.15^{bc}	26.33±0.88^{c}	28.80±0.12^{b}	24.01	0.00	
Phosphate (mg/l)	2.37±0.09^{a}	2.20±0.00^{b}	1.05±0.06^{d}	1.25±0.03^{c}	0.77±0.02^{e}	199.63	0.00	5
Oil and grease (mg/l)	0.77±0.12^{cd}	0.97±0.03^{c}	3.93±0.12^{a}	0.63±0.09^{d}	1.97±0.03^{b}	4.51	0.00	10

Means with different superscripted alphabets across the row are significantly ($P \leq 0.05$) different. Values are expressed as means±SE (Standard error of means). A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, FEMNV=Federal Ministry of Environment, Nigeria, µS/cm=Micro siemens per centimeter, mg/l=Milligram per liter.

4.2 Heavy Metal Contents of the Refinery Effluent and Water Samples from Romi River

Table 4.2 shows the heavy metal contents of the effluent and water samples from Romi River. The concentrations of lead at the five sampling sites, A (0.11mg/l), B (0.15mg/l), C (0.40mg/l), D (0.09mg/l) and E (0.35mg/l), were above the permissible limit (0.01mg/l) of FEMNV. It was observed that nickel concentrations at sampling site A (0.22mg/l), B (0.08mg/l), C (0.08mg/l) and E (0.11mg/l) were higher compared to the standard limit (0.07mg/l) of FEMNV. Cadmium concentration of the study site revealed that Cd concentration of site E (0.06mg/l) was high compared to standard limit (0.03mg/l) set by FEMNV, Nigeria. However, significant difference ($P<0.05$) exists between the means of metal concentrations and the sampling sites.

Table 4.2: Heavy Metal Contents of the Refinery Effluent and Water Samples from Romi River

Heavy Metals	Sampling points					F value	P value	Permissible limit FMENV
	A	B	C	D	E			
Lead (mg/l)	0.11 ± 0.00^{d}	0.15 ± 0.01^{c}	0.40 ± 0.01^{a}	0.09 ± 0.02^{d}	0.35 ± 0.02^{b}	201.47	0.00	0.01
Nickel (mg/l)	0.22 ± 0.01^{a}	0.08 ± 0.01^{cd}	0.08 ± 0.01^{cd}	0.06 ± 0.01^{d}	0.11 ± 0.01^{b}	67.97	0.00	0.07
Cadmium(mg/l)	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.06 ± 0.00^{a}	35.00	0.00	0.03

Means with different superscripted alphabets across the row are significantly ($P\leq0.05$) different. Values are expressed as means±SE (Standard error of means). A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, FEMNV=Federal Ministry of Environment, Nigeria, mg/l=Milligram per liter.

4.3 Characterization of the Fungal Species Isolated from the Study Site

The cultural and microscopic characteristics of the fungal isolates from the various sampling sites (A, B, C, D and E) are presented in Table 4.3 and shown pictorially in Plates I to XV. The fungal genera identified on potato dextrose agar, were *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia*, *Trichoderma*, *Nigrospora*, *Microsporum*, *Rhizoctonia*, *Trichophyton*, *Geotrichum* and *Chaetophoma*. *Aspergillus* spp. gave distinct cultural and microscopic characteristics depending on its species which were identified as *Aspergillus flavus* (Plate I), *Aspergillus niger* (Plate II), *Aspergillus fumigatus* (Plate III), *Aspergillus carbonarius* (Plate IV) and *Aspergillus glaucus* (Plate V). Microscopic characteristic common among these species of *Aspergillus* is that their reproductive hyphae sprang from foot cells; The *Penicillium* spp. (Plate VI), appeared as bluish-green colony, septate hyphae, with unbranched conidiophores and flask shaped phialides.

Curvularia spp. (Plate VII), appeared as brown colony and brown knobby conidiophores containing 4 cells in which the swollen central cells gave it a curved appearance; *Fusarium* spp. (Plate VIII), a pink centered white colony had a canoe shaped macroconidia with 4-5 cells; *Trichoderma* spp. (Plate IX), appeared as a white cottony colony with green patches, short conidiophores and flask shaped clustered oval conidia. Among other characteristics *Trichophyton* spp. (Plate X), appeared as a white colony with chlamydoconidia and irregular knobby hyphae; *Nigrospora* spp. (Plate XI), appeared as a woolly colony with swollen ampliform conidiophores; *Microsporum* spp. (Plate XII), had white cottony colony and branched. *Chaetophoma* spp. (Plate XIII) appeared as a grayish brown colony with globose pycnidia; *Rhizoctonia* spp. (Plate XIV), appeared as a white cottony colony with constricted branched hyphae; *Geotrichum* spp. (Plate XV), a black colony had rectangular arthroconidia.

Table 4.3: Characterization of the Fungal Isolates

Cultural Characteristics	Microscopic Characteristics	Inferences
Green colony with granular surface and a brown reverse coloration on PDA.	Septate hyphae, hyaline and coarsely rough conidiophores. Radial and biseriate conidial head. The phialides point out in all directions.	*Aspergillus flavus*
Black colony with granular surface and black reverse	Septate hyphae. Dark brown large globose conidial heads. Hyaline smooth-walled conidiophores which turn dark towards the vesicle. Conidial heads are biseriate.	*Aspergillus niger*
Gray colony with granular surface, white edges and a black reverse coloration on PDA	Septate hyphae and short smooth-walled conidiophores. Typical columnar, uniseriate conidial heads and conidial shaped terminal vesicle.	*Aspergillus fumigatus*
Black colony with crenated granular surface, white edges and brown reverse coloration on PDA	Septate hyphae, long stipe, black rough-walled conidia with spikes. Radiate and biseriate conidial head.	*Aspergillus carbonarius*
Green colony with yellow areas and yellow reverse coloration on PDA.	Septate hyphae, Phialides are uniseriate, radiate to very loosely columnar and cover the entire vesicle.	*Aspergillus glaucus*
Bluish-green colony with cottony surface, white border and a brown reverse coloration on PDA	Septate hyphae with unbranched conidiophores and secondary branches (metulae). The metulae bear flasked shaped phialides with unbranched chains of smooth and round conidia.	*Penicillium* sp.
Velvety-brown colony with pinkish gray woolly surface and a brown reverse coloration on PDA.	Brown septate hyphae. Conidiophores are simple and Knobby at point of conidium formation (geniculate growth). Conidia are large and contain 4 cells. The swelling of the central cell gives the conidium a curved appearance.	*Curvularia* sp.

Table 4.3: Characterization of the Fungal Isolates Cont'd

Cultural Characteristics	Microscopic Characteristics	Inferences
Pink centered white colony with cottony surface and a brown reverse coloration on PDA	Septate hyphae, canoe shaped macroconidia. The macroconidia are multiseptate which contain 4-5 cells. Presence of macroconidia attached to the conidiophores.	*Fusarium* sp.
Black colony with cottony surface and a brown reverse coloration on PDA.	Septate hyphae, Absence of blastoconidia and coniphores. Hyphae segment into rectangular arthroconidia which vary in length and roundness of their ends.	*Geotrichum* sp.
Round, rapidly growing wooly colony with black areas and black reverse coloration on PDA.	Septate hyaline hyphae. The conidiogenious cells on the conidiophores are inflated, swollen and ampliform in shape.	*Nigrospora* sp.
White colony with cottony surface and white reverse coloration on PDA.	Light brown septate hyphae branched at 90^{o} angles.and have constriction at the base of hyphal branching. Presence of mycelium and sclerotia.	*Rhizoctonia* sp.
White colony with folded cottony surface, creamy colored spores and a yellowish brown reverse coloration on PDA.	Irregular knobby septate hyphae with swollen tips that resemble nail heads. Presence of chlamydoconidia	*Trichophyton* sp.
White colony with cottony surface and cream reverse coloration on PDA.	Septate hyphae. Hyphae are irregularly branched, clubbed and fragmented and have intercalary chlamydoconidia-like cells.	*Microsporum* sp.
White colony with cottony surface and green patches which spread from the centre of the colony to the margin and yellow reverse coloration on PDA.	Septate hyphae, short conidiophores which are flask shaped and clustered together at the end of each phialide.	*Trichoderma* sp.
Grayish-brown colony with cottony surface and black reverse coloration on PDA.	Septate hyaline hyphae. Small, globose and irregular dark dense pycnidia without ostiole.	*Chaetophoma* sp.

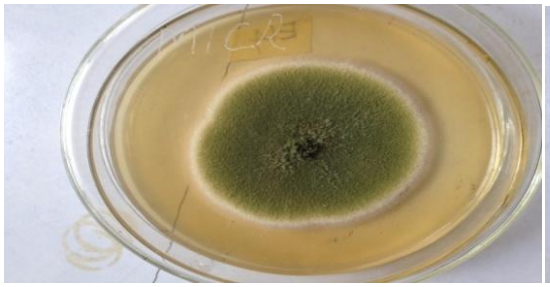


Plate Ia: ***Aspergillus flavus***

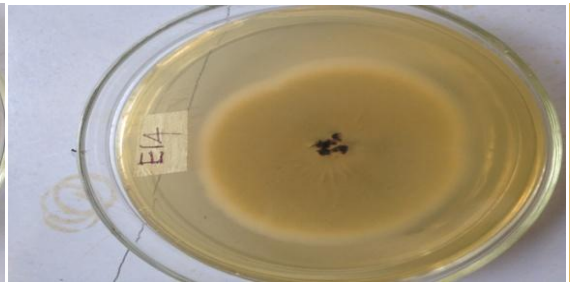


Plate Ib: ***Aspergillus flavus***

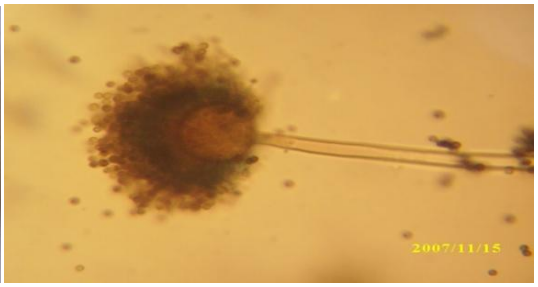


Plate Ib: ***Aspergillus flavus***

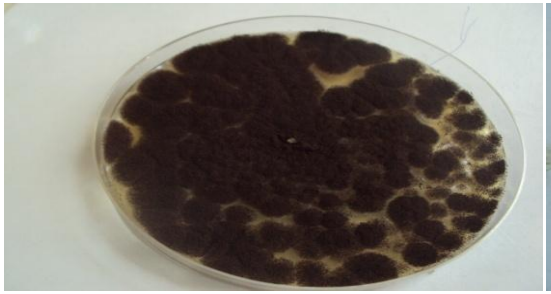


Plate IIa: ***Aspergillus niger***

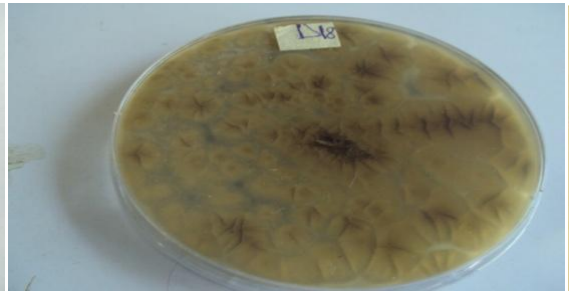


Plate IIb: ***Aspergillus niger***

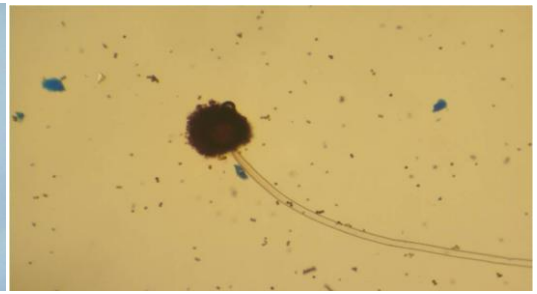


Plate IIc: ***Aspergillus niger***

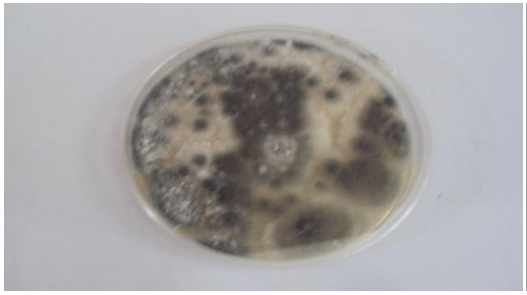


Plate IIIa: ***Aspergillus fumigatus***

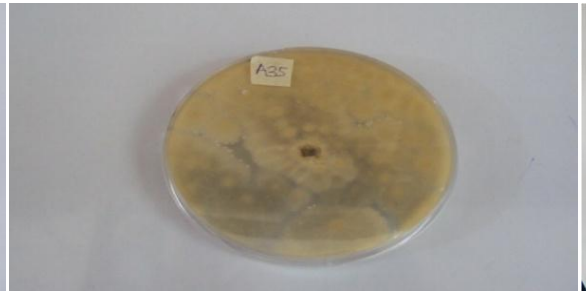


Plate IIIb: ***Aspergillus fumigatus***

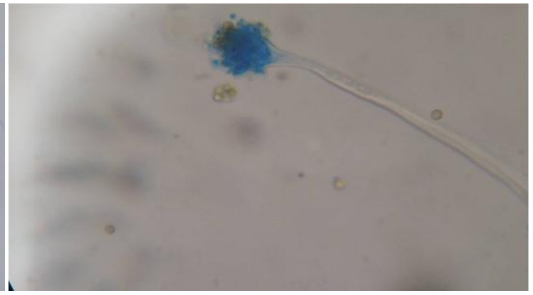
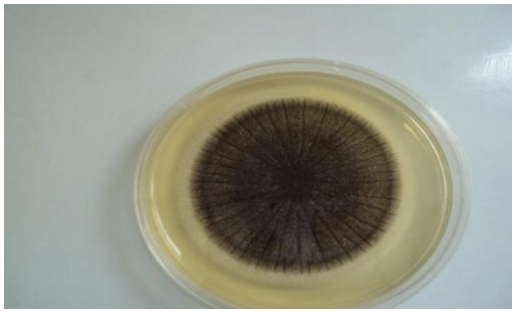


Plate IIIc: ***Aspergillus fumigatus***

a=Surface characteristics; b=Reverse side; c=Microscopic characteristics



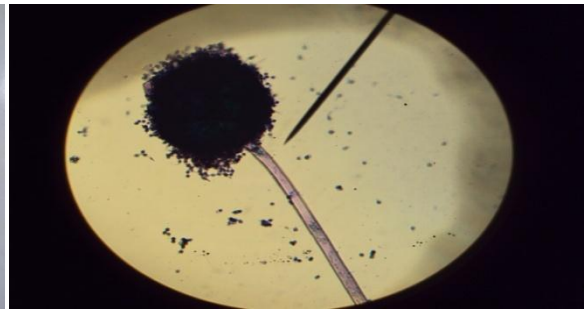
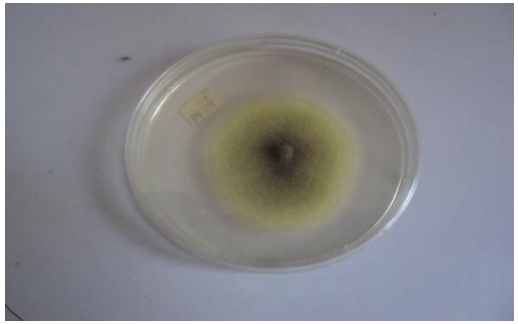
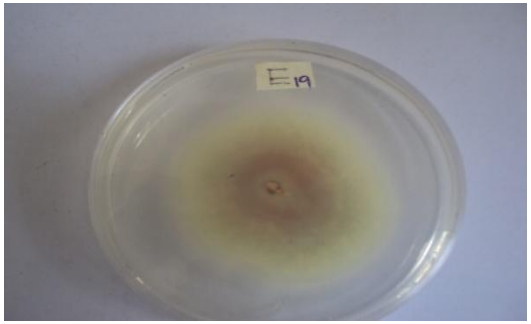



Plate IVa: ***Aspergillus carbonarius*** **Plate IVb:** ***Aspergillus carbonarius*** **Plate IVc:** ***Aspergillus carbonarius***

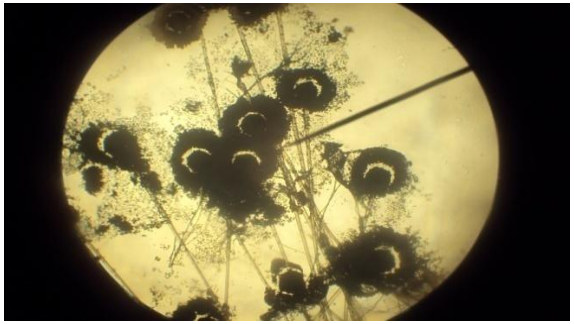


Plate Va: ***Aspergillus glaucus*****Plate Vb:** ***Aspergillus glaucus*** **Plate Vc:** ***Aspergillus glaucus***






Plate VIa: ***Penicillium*** **sp.** **Plate VIb:** ***Penicillium*** **sp.** **Plate VIc:** ***Penicillium*** **sp.**

a=Surface characteristics, b=Reverse side, c=Microscopic characteristics

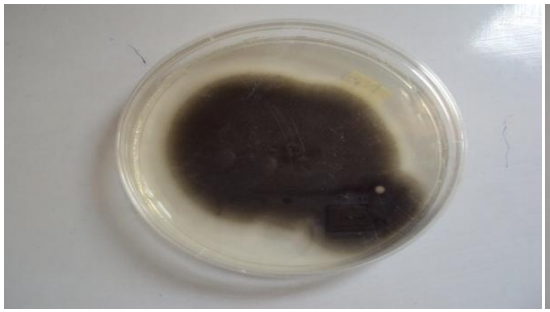
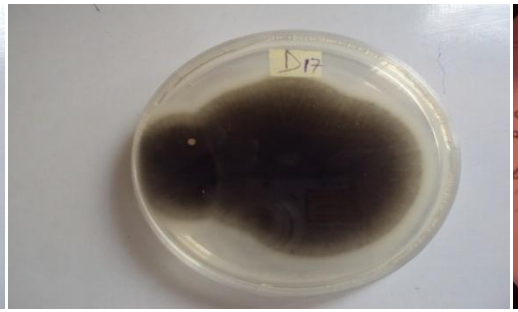





Plate VIIa: *Curvularia* sp.Plate VIIb: *Curvularia* sp. **Plate VIIc: *Curvularia* sp.**

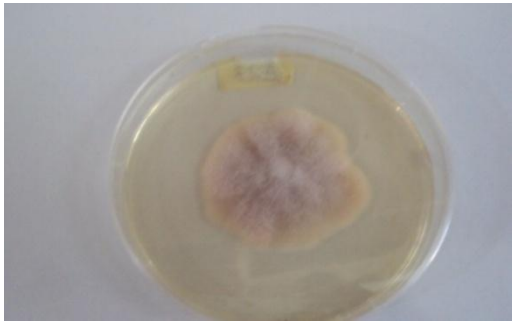
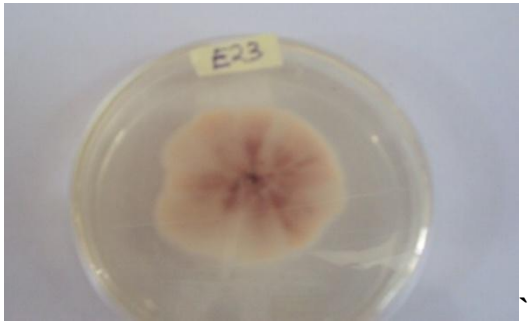
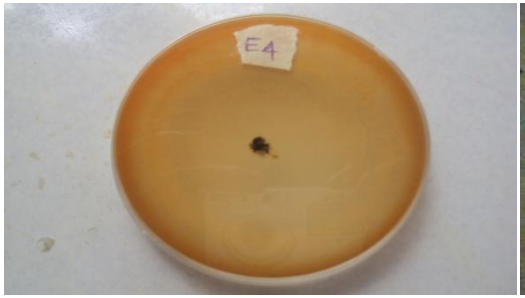





Plate VIIIa: *Fusarium* sp. **Plate VIIIb: *Fusarium* sp.** **Plate VIIIc: *Fusarium* sp.**

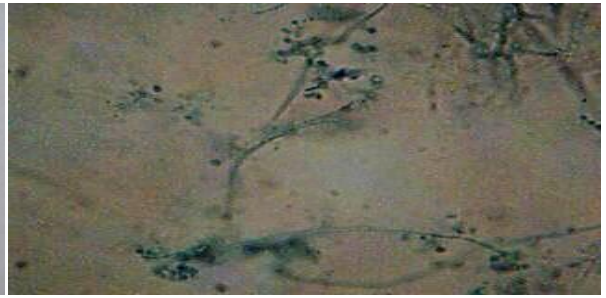
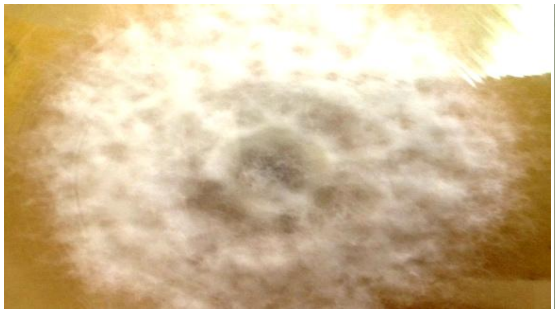
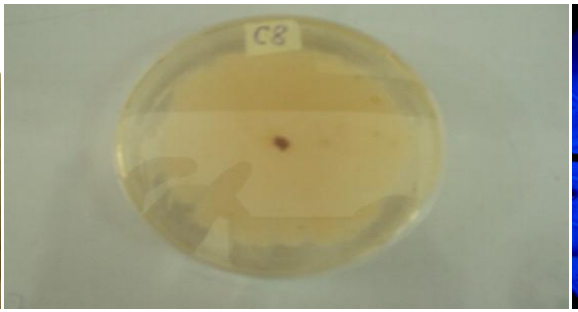


Plate IXa: *Trichoderma* sp. **Plate IXb: *Trichoderma* sp.** **Plate IXc: *Trichoderma* sp.**

a=Surface characteristics, b=Reverse side, c=Microscopic characteristics

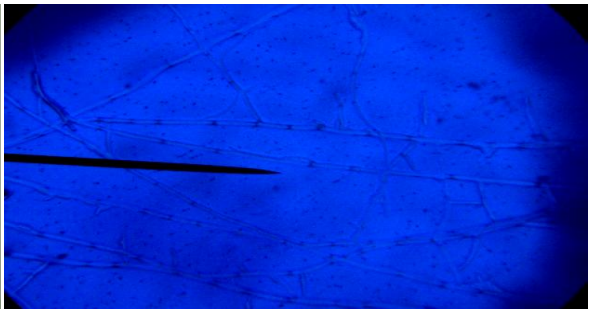


Plate Xa: ***Trichophyton*** **sp. Plate Xb:** ***Trichophyton*** **sp.** **Plate Xc:** ***Trichophyton*** **sp.**



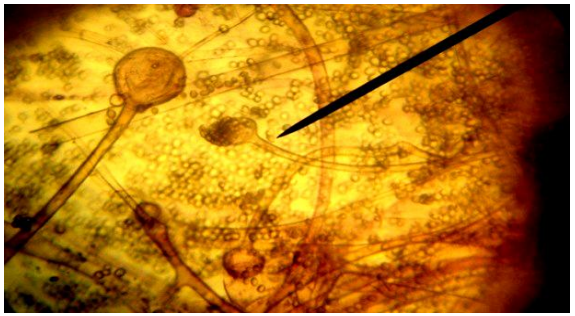
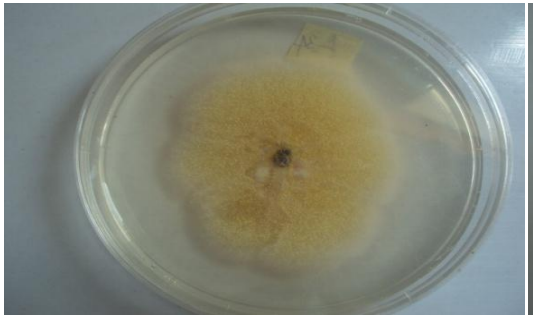
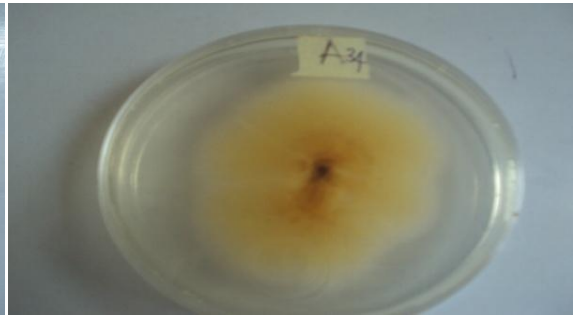



Plate XIa ***Nigrospora*** **sp.** **Plate XIb:** ***Nigrospora*** **sp.** **Plate XIc:** ***Nigrospora*** **sp**

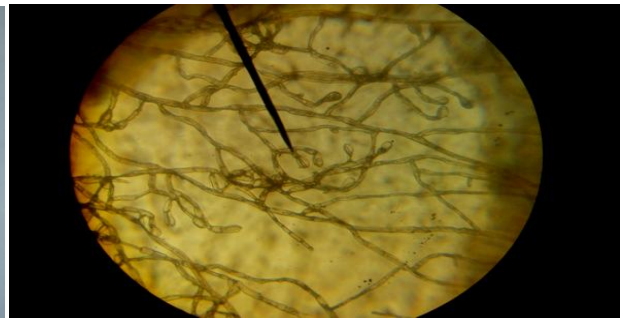
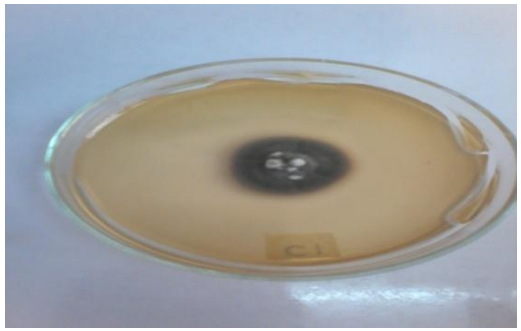
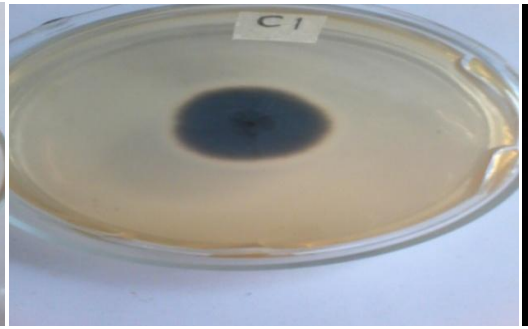


Plate XIIa: ***Microsporum*** **sp.** **Plate XIIb:** ***Microsporum*** **sp.** **Plate XIIc:** ***Microsporum*** **sp.**

a=Surface characteristics, b=Reverse side, c=Microscopic characteristics

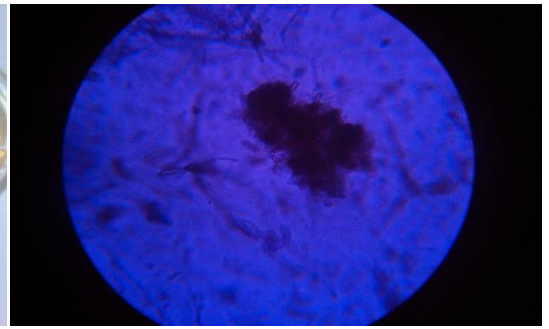


Plate XIIIa: *Chaetphoma* sp. **Plate XIIIb: *Chaetphoma*sp.** **Plate XIIIc: *Chaetophoma* sp**

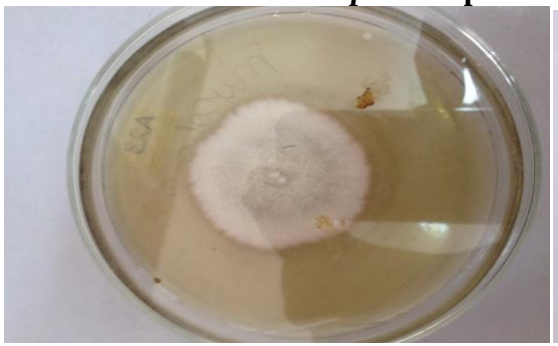
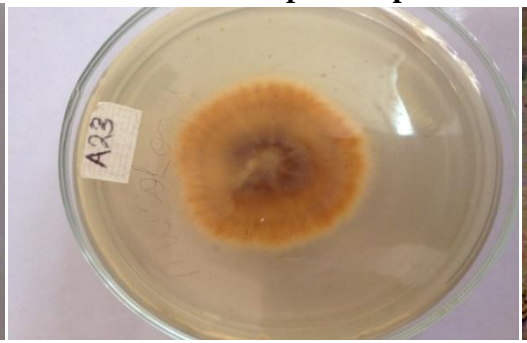
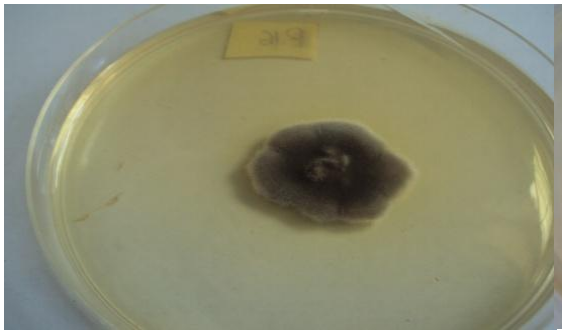





Plate XIVa: *Rhizoctonia* sp.Plate XIVb: *Rhizoctonia* sp. **Plate XIVb: *Rhizoctonia* sp.**



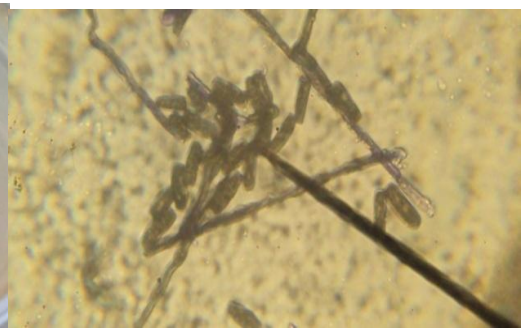


Plate XVa: *Geotrichum* sp. Plate XVb: *Geotrichum* sp. Plate XVc: *Geotrichum* sp.

a=Surface characteristics, b=Reverse side, c=Microscopic characteristics

4.4 Frequency of Occurrence of fungal species isolated from the Study Sites

A total of 133 fungal isolates belonging to 15 species were obtained from the study site (Table 4.4) of which *Aspergillus flavus* had the highest total occurrence with 24(18.05%), followed by *Aspergillus niger* and *Penicillium* spp. which had the same occurrence of 17(12.78%) each. *Aspergillus glaucus* and *Trichophyton* spp. had the least and equal occurrence of 1(0.75%) each, in the samples analyzed. *Geotrichum* sp., *Nigrospora* spp. and *Chaetophoma* spp. were not part of the fungal flora upstream of the discharge point. Similarly, *Trichoderma* sp. and *Chaetophoma* sp. were not detected in water samples obtained from the effluent discharge point. Also, along the sampling sites (Table 4.4), the highest 38(28.57%) occurrence was observed at untreated waste water channel and the least 18(13.53%), was observed at the Romi river downstream surface water.

Data obtained on the distribution of *Aspergillus* species (Table 4.5) from the study sites revealed that only *A.flavus* and *A. niger* form part of the fungal flora of the five sampling sites. *A.fumigatus* was completely absent in the waste oil retention pond and the discharge point into Romi River. Also, *A. carbonarius* was absent in waste oil retention pond.

Table 4.4: Percentage Occurrence of Fungi Isolated from the Study Site

Fungal Isolates	Sampling Sites No. (%)					TOTAL (%)
	A (%)	B (%)	C (%)	D (%)	E (%)	
Aspergillus flavus	6 (4.51)	3(2.26)	6(4.51)	3(2.26)	6(4.51)	24(18.05)
Aspergillus niger	6(4.51)	4(3.01)	1(0.75)	4(3.01)	2(1.50)	17(12.78)
Penicillium spp.	7(5.26)	3(2.26)	2(1.150)	1(0.75)	4(3.01)	17(12.78)
Curvularia spp.	2(1.50)	7(5.26)	5(3.76	2(1.50)	0(0.00)	16(12.03)
Fusarium spp.	3(2.26)	5(3.76)	2(1.150)	2(1.50)	1(0.75)	13(9.77)
Aspergillus carbonarius	2(1.50)	0(0.00)	3(2.26)	1(0.75)	2(1.50)	8(6.02)
Rhizoctonia spp.	2(1.50)	0(0.00)	2(1.150)	2(1.50)	1(0.75)	7(5.26)
Microsporum spp.	2(1.50)	0(0.00)	4(3.01)	1(0.75)	0(0.00)	7(5.56)
Geotrichum spp.	0(0.00)	2(1.56)	3(2.26)	0(0.00)	1(0.75)	6(4.51)
Trichoderma spp.	2(1.50)	0(0.00)	0(0.00)	2(1.50)	2(1.50)	6(4.51)
Aspergillus fumigatus	2(1.50)	0(0.00)	1(0.75)	0(0.00)	2(1.50)	5(3.76)
Nigrospora spp.	2(1.50)	0(0.00)	1(0.75)	0(0.00)	0(0.00)	3(2.26)
Chaetophoma spp.	1(0.75)	0(0.00)	0(0.00)	0(0.00)	1(0.75)	2(1.50)
Aspergillus glaucus	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.75)	1(0.75)
Trichophyton spp.	1(0.75)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.75)
TOTAL	**38(28.57)**	**24(18.05)**	**30(22.45)**	**18(13.53)**	**23 (17.29)**	**133(100)**

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River

Table 4.5: Occurrence of *Aspergillus* species in Samples of Raw Effluent, Waste Oil Retention Pond and Romi River

Sampled Sites	No. of Isolates of (per 0.1ml of sample)			
	A. flavus	***A. niger***	***A. fumigatus***	***A. carbonarius***
Untreated waste water channel	4 (++)	7 (+++)	2 (+)	2 (+)
Waste oil retention pond	2 (+)	3 (++)	-	-
Discharge point into Romi River	3 (++)	1 (+)	1 (+)	3 (++)
Upstream of discharge point	3 (++)	2 (+)	-	1 (+)
Downstream of discharge point	4 (++)	3 (++)	2 (+)	2 (+)

(+++)=High, (++) =Moderate, (+) =Low, (-) =No growth

4.5 Heavy Metal Resistance study of the Fungal Isolates to Pb, Ni and Cd

4.5.1 Heavy metal resistance of individual fungal isolates

Data gathered from this study strongly suggest that resistance to the toxicity of Pb, Ni and Cd is common among members of mycoflora of petroleum refinery effluents and rivers impacted by the effluents over a long time. 52 out of the 133 fungal isolates were resistant to the test heavy metals (Table 4.6a-4.6e). It was observed that of the 28 isolates of *Aspergillus* species tested, 4 out of 10 of *A. niger* (Table 4.6a) and *A. flavus* (Table 4.6b), 1 out of 3 of *A. fumigatus*, 2 out of 4 of *A.carbonarius* (Table 4.6c) resisted and grew in the presence of 5 to 15µg/ml of these metals. Similar level of resistance to Pb, Ni and Cd was recorded among 5 out of 9 isolates of *Penicillium* spp. (Table 4.6d), 2 out 4 of *Nigrospora* spp., 1 out of 3 of *Curvularia* sp., 1 out of 2 of *Trichoderma* sp., and *Microsporum* sp. (Table 4.6e).

However, relative analysis of the data revealed that, genera and species of fungi vary in their capacity to resist Pb, Ni and Cd. For instance, *A. niger* (Table 4.6a), *A. fumigatus* (Table 4.6c)and *A. carbonarius* (Table 4.6c)appeared to be the most resistant to Pb followed by *Penicillium* sp (Table 4.6d) and *Fusarium* sp. (Table 4.6e) *A. flavus* (Table 4.6b), *Curvularia* sp (Table 4.6e), *Nigrospora* sp (Table 4.6e) and other resistant fungal isolates were suppressed by the same metal. On the other hand, *A. niger, A. carbonarius* and *Fusarium* sp. were found to be the most resistant to Ni while Cd suppressed the growth of all test isolates except *Penicillium* sp. and *A. carbonarius*.

Table 4.6a: Resistance to Pb, Ni and Cd by *Aspergillus niger* Isolated from Refinery Effluent and Effluent Impacted River

Coded Fungal Isolates Tested	Source of Isolates	Yield of Dry Mycelial Biomass (mg) in the Presence of								
		Pb(µg/ml)			Ni(µg/ml)			Cd(µg/ml)		
		5	10	15	5	10	15	5	10	15
Aspergillus niger A1	Site A	96	85	80	107	98	93	83	76	73
Aspergillus niger A2	Site A	119	113	105	127	117	106	90	84	82
Aspergillus niger B1	Site B	60	56	48	65	53	49	37	29	22
Aspergillus niger E1	Site E	66	57	50	46	38	30	40	28	16
Aspergillus niger D1	Site D	48	40	25	54	42	30	25	17	-
Aspergillus niger C2	Site C	34	23	-	77	58	45	31	25	19
Aspergillus niger B2	Site B	96	86	-	74	63	-	46	38	34
Aspergillus niger E2	Site E	72	64	-	70	64	44	54	48	-
Aspergillus niger C1	Site C	83	67	50	60	40	27	-	-	-
Aspergillus niger D2	Site D	38	25	-	60	46	39	-	-	-

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, (-)=No growth.

Table 4.6b: Resistance to Pb, Ni and Cd by *Aspergillus flavus* Isolated from Refinery Effluent and Effluent Impacted River

Coded Fungal Isolates Tested	Source of Isolates	Yield of Dry Mycelial Biomass (mg) in the Presence of								
		Pb(µg/ml)			Ni(µg/ml)			Cd(µg/ml)		
		5	10	15	5	10	15	5	10	15
Aspergillus flavus A1	Site A	45	40	39	39	38	36	40	36	32
Aspergillus flavus A2	Site A	69	60	57	75	72	71	95	88	86
Aspergillus flavus C2	Site C	56	41	29	70	64	34	40	30	19
Aspergillus flavus E1	Site E	50	42	21	69	63	60	47	40	36
Aspergillus flavus C1	Site C	53	31	16	79	65	46	39	24	-
Aspergillus flavus E2	Site E	52	44	-	33	28	-	29	26	-
Aspergillus flavus D1	Site D	41	29	17	89	77	70	-	-	-
Aspergillus flavus D2	Site D	43	31	22	35	26	12	-	-	-
Aspergillus flavus B2	Site B	39	28	-	-	-	-	38	33	-
Aspergillus flavus B1	Site B	39	34	29	-	-	-	-	-	-

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, (-)=No growth.

Table 4.6c: Resistance to Pb, Ni and Cd by other *Aspergillus* species Isolated from Refinery Effluent and Effluent Impacted River

Coded Fungal Isolates Tested		Source of Isolates	Yield of Dry Mycelial Biomass (mg) in the Presence of								
			Pb(µg/ml)			Ni(µg/ml)			Cd(µg/ml)		
			5	10	15	5	10	15	5	10	15
Aspergillus fumigatus	A	Site A	42	30	25	73	69	63	76	75	75
Aspergillus fumigatus	E	Site E	79	72	66	73	62	55	-	-	-
Aspergillus fumigatus	C	Site C	70	63	-	75	68	-	66	60	-
Aspergillus carbonarius	A	Site A	88	73	62	97	93	81	76	75	75
Aspergillus carbonarius	E	Site E	85	80	73	70	69	54	57	57	51
Aspergillus carbonarius	C	Site C	34	23	-	77	58	45	31	25	19
Aspergillus carbonarius	D	Site D	47	37	29	49	38	-	-	-	-
Aspergillus glaucus	E	Site E	37	32	-	36	-	-	38	28	20

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, (-)=No growth

Table 4.6d: Resistance to Pb, Ni and Cd by *Penicillium* species Isolated from Refinery Effluent and Effluent Impacted River

Coded Fungal Isolates Tested	Source of Isolates	Yield of Dry Mycelial Biomass (mg) in the Presence of								
		Pb(µg/ml)			Ni(µg/ml)			Cd(µg/ml)		
		5	**10**	**15**	**5**	**10**	**15**	**5**	**10**	**15**
Penicillium sp.A1	Site A	77	74	68	67	61	56	84	80	73
Penicillium sp. A2	Site A	51	42	33	22	17	13	45	41	32
Penicillium sp. B2	Site B	30	25	13	36	21	14	60	49	44
Penicillium sp. C1	Site C	15	56	41	68	59	42	59	52	39
Penicillium sp. E2	Site E	72	70	66	74	72	66	60	57	51
Penicillium sp. C2	Site C	40	29	16	59	50	40	65	53	-
Penicillium sp. B1	Site B	80	74	-	86	75	72	31	25	-
Penicillium sp.D1	Site D	35	27	-	27	19	-	16	14	11
Penicillium sp. E1	Site E	90	80	-	89	80	72	79	68	63

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, (-)=No growth

Table 4.6e: Resistance to Pb, Ni and Cd by other Fungi Isolated from Refinery Effluent and Effluent Impacted River

Coded Fungal Isolates Tested	Source of Isolates	Yield of Dry Mycelial Biomass (mg) in the Presence of								
		Pb(µg/ml)			Ni(µg/ml)			Cd(µg/ml)		
		5	10	15	5	10	15	5	10	15
Curvularia sp. A	Site A	41	40	33	54	54	49	47	39	37
Curvularia sp. B	Site B	47	30	20	25	20	-	19	14	11
Curvularia sp. C	Site C	40	29	16	59	50	40	65	53	-
Curvularia sp. D	Site D	-	-	-	36	-	-	-	-	-
Fusarium sp. A	Site A	77	71	64	82	75	-	11	12	-
Fusarium sp. B	Site B	54	49	40	62	50	42	43	33	26
Fusarium sp. D	Site D	28	20	-	50	55	44	-	-	-
Nigrospora sp. A	Site A	30	20	15	33	26	15	19	15	14
Nigrospora sp. C	Site C	41	29	-	60	43	-	56	45	-
Trichoderma sp. A	Site A	56	50	40	89	81	68	62	16	15
Trichoderma sp. E	Site E	63	58	47	98	87	78	-	-	-
Microsporum sp. C	Site C	88	58	48	83	67	48	45	32	24
Geotrichum sp. C	Site C	59	56	50	-	-	-	48	50	44
Rhizoctonia sp. C	Site C	66	49	-	39	27	19	52	30	21
Chaetophoma sp. E	Site E	39	29	25	26	27	-	20	-	-

A=Untreated waste water channel, B=Waste oil retention pond, C=Discharge point, D=Upstream of Romi River, E=Downstream of Romi River, (-)=No growth

4.5.2 Responses of the fungal isolates to the same concentrations of the test heavy metals

The responses of the test isolates were also found to vary when grown at the same concentrations of the test metals. At 5µg/ml (Figure 4.1), *A.niger, A. fumigatus, Penicillium* sp., *Fusarium* sp. and *A. flavus* were the most resistant to Pb. Similar response was also noted for the same level of concentration of Ni. Also noted in this study, Cd was found to suppress the growth of all isolates except *Trichoderma* sp. and *Rhizoctonia* sp. and *Penicillium* sp. at 5µg/ml concentration.

Increasing the concentration of the test metals to 10µg/ml yielded the same trend of resistance among the isolates to Pb, Ni and Cd (Figure 4.2). However the mean yield of dry biomass was generally lower at 10µg/ml than at 5µg/ml.

Increasing the levels of the test metals to 15µg/ml resulted in significant reduction in the biomass yield of all the isolates tested (Figure 4.3). Ni appeared to be better resisted at this concentration compared to Pb and Cd.

4.5.3 Tolerance index of the fungal isolates to test heavy metals

Figures 4.4a-4.4c, depict the tolerance index of the resistant fungal isolates to the test heavy metals. In Figure 4.4a, *Aspergillus fumigatus* had the highest tolerance index (2.4) to Pb; this was followed by *Aspergillus niger* with tolerance index of 1.7. *Chaetophoma* sp. had the least tolerance index value of 0.09 to Pb. In Figure 4.4b, *Fusarium* sp. had the highest (1.16) tolerance index to Ni, followed by *Penicillium* sp. with a value of 1.04. *Geotrichum* sp. ranked the least (0.12) tolerance to Ni. In Figure 4.4c, *Penicillium* sp. ranked the highest (1.81) in tolerance index of Cd. *Chaetophoma* sp. ranked the lowest (0.1) tolerance to Cd.

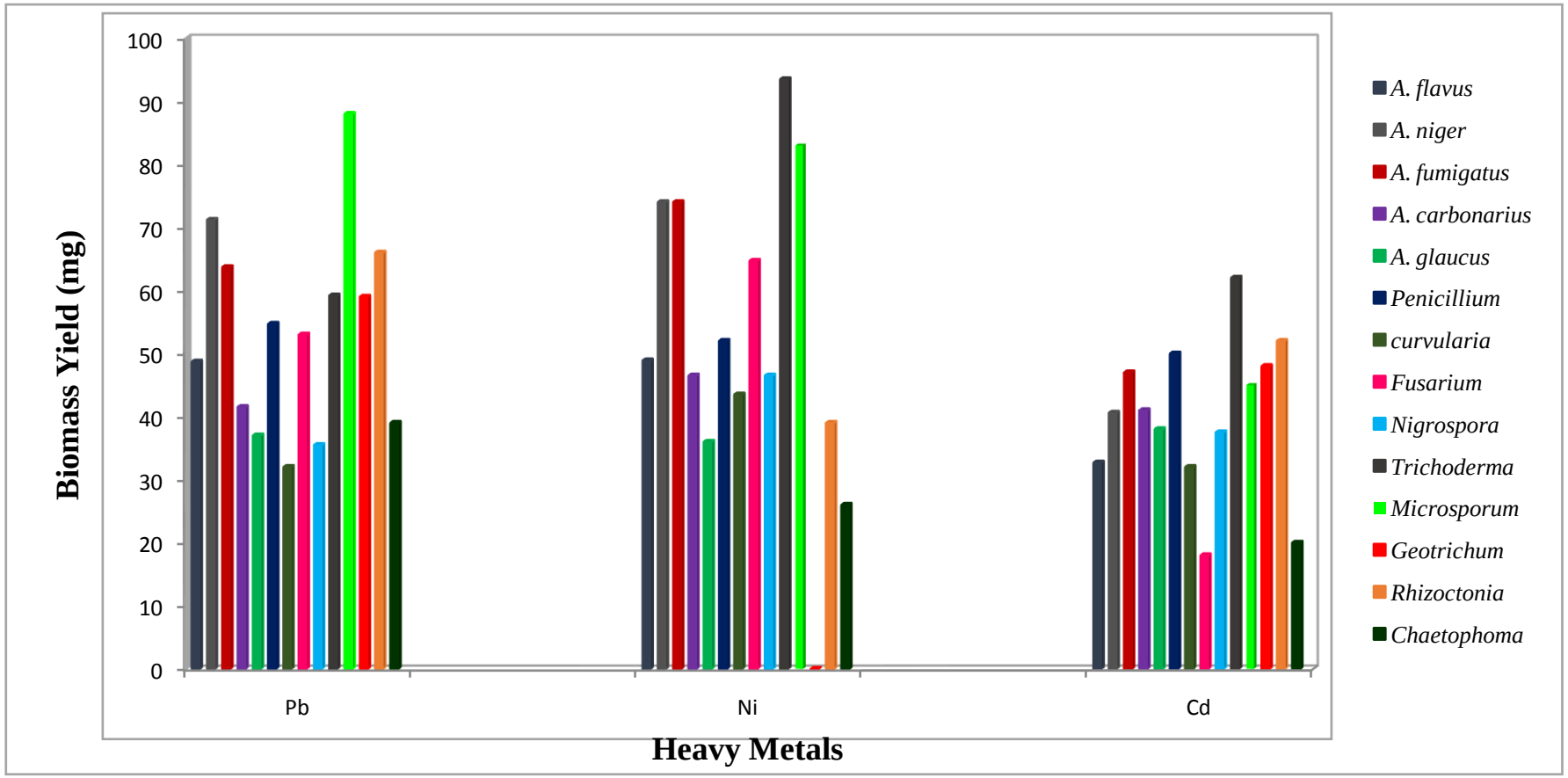


Figure 4.1: Mean Biomass Yield of the Test Fungal Isolates to 5 µg/ml of Heavy Metals

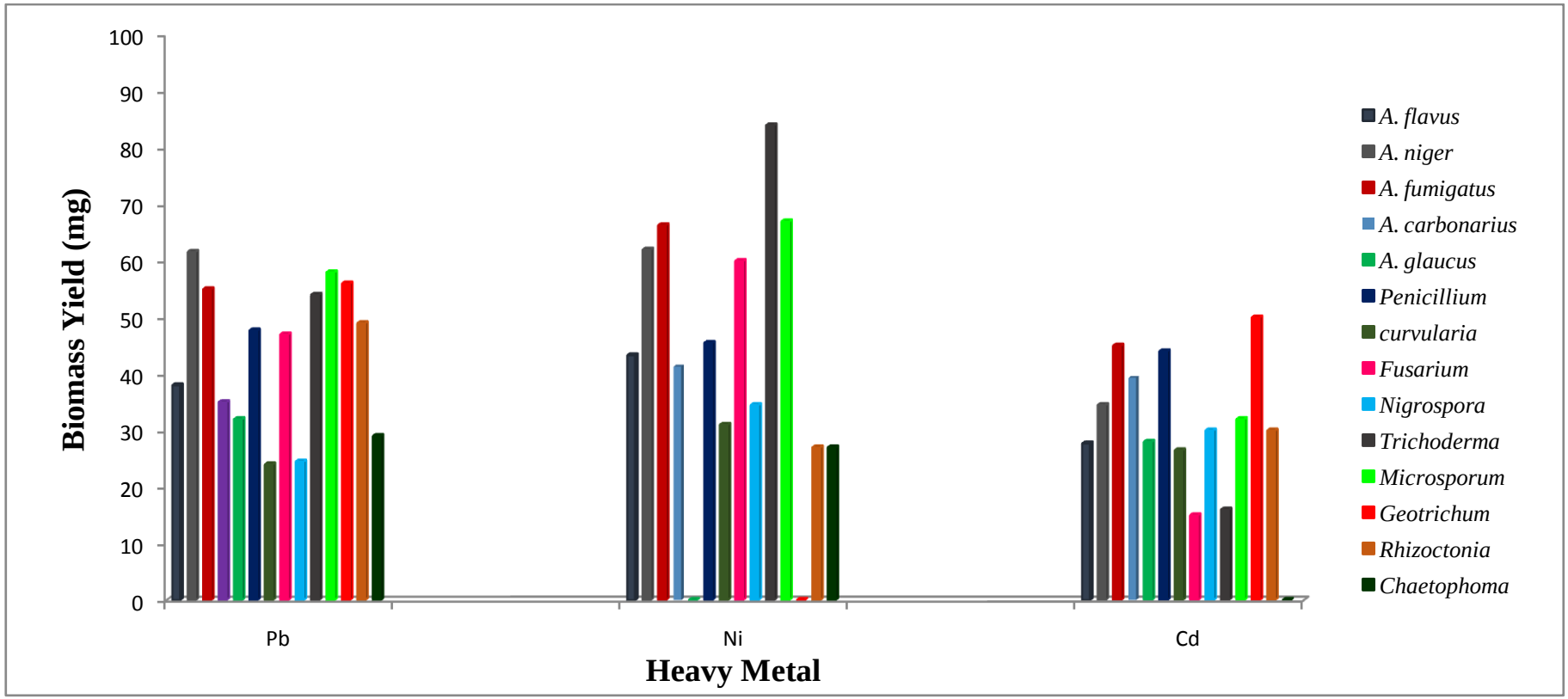


Figure 4.2: Mean Biomass Yield of the Test Fungal Isolates to 10µg/ml of Heavy Metals

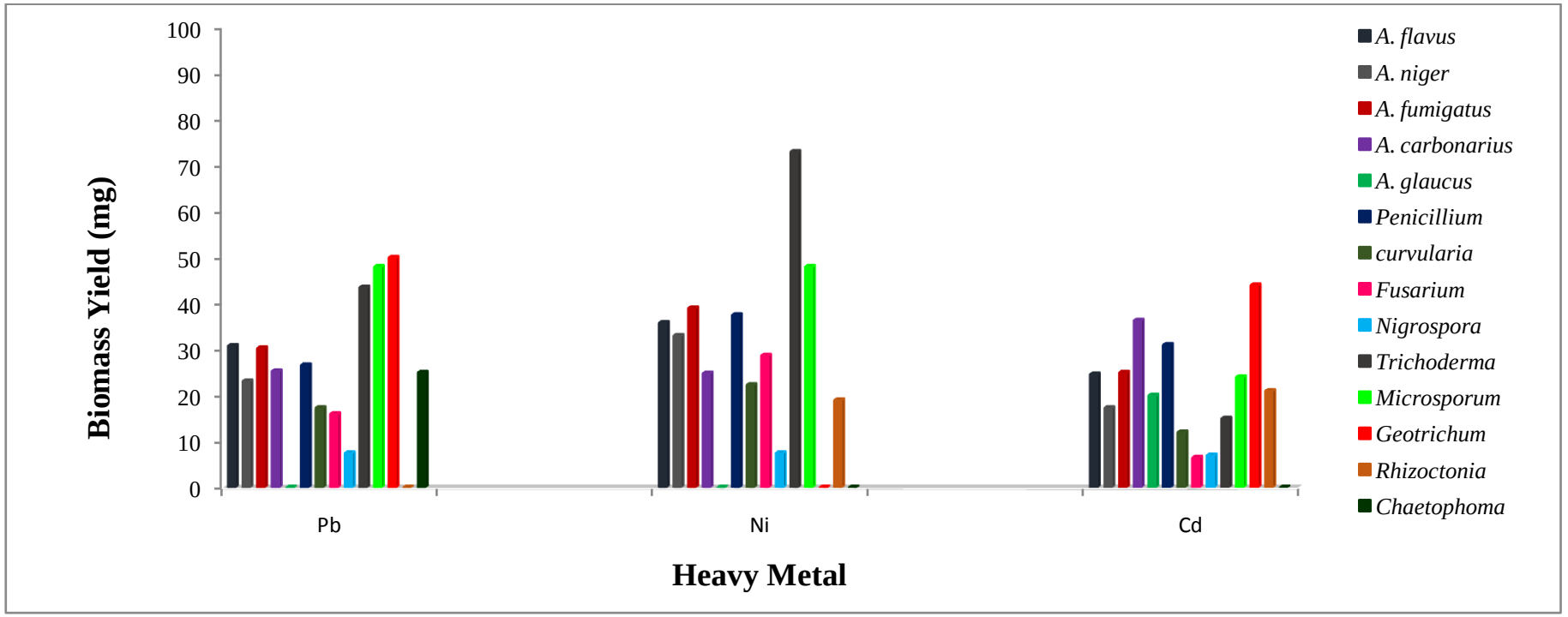


Figure 4.3: Mean Biomass Yield of Test Fungal Isolates to 15µg/ml of Heavy Metals

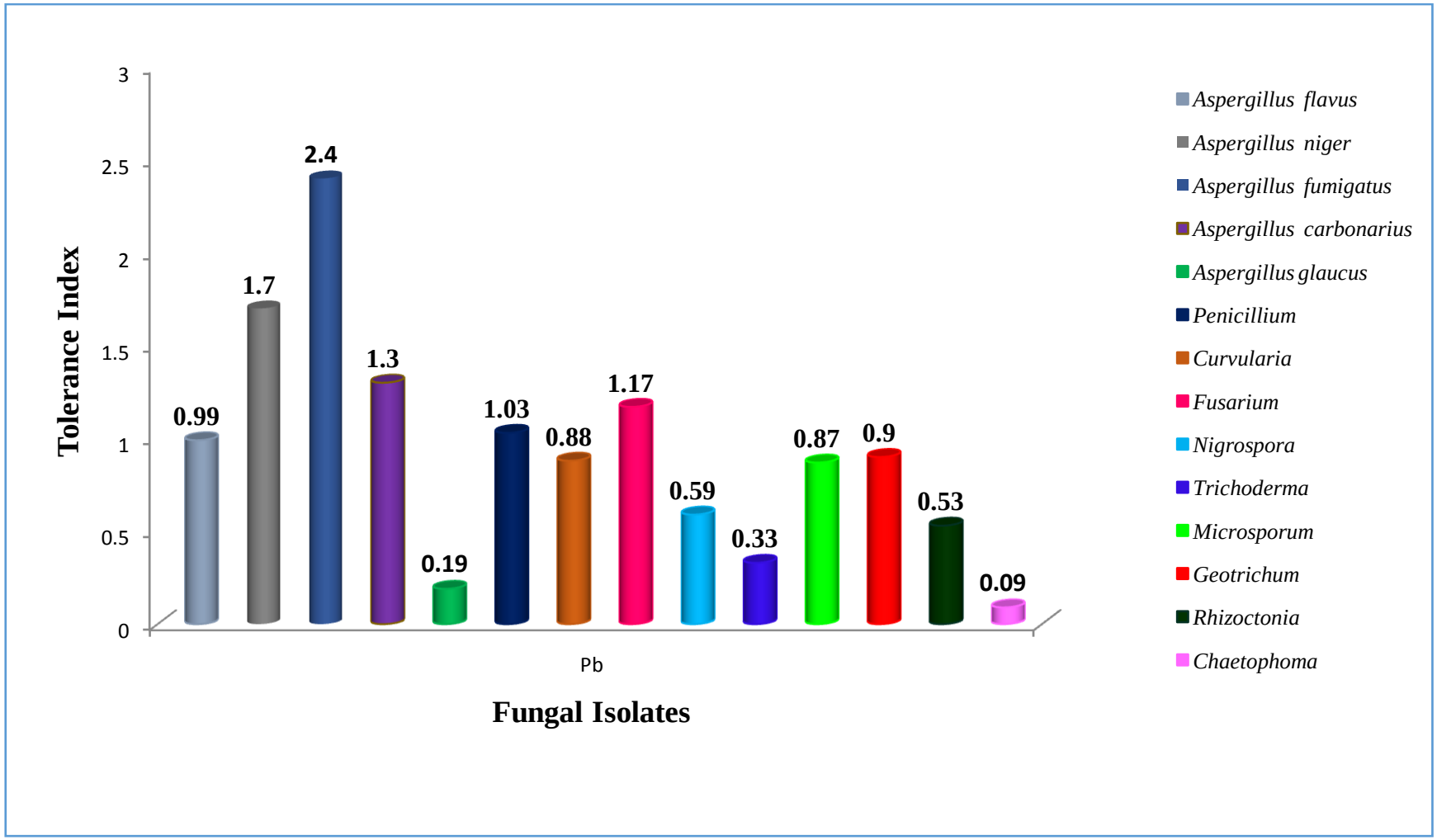


Figure 4.4a: Tolerance Indices of Test Fungal Isolates to Lead

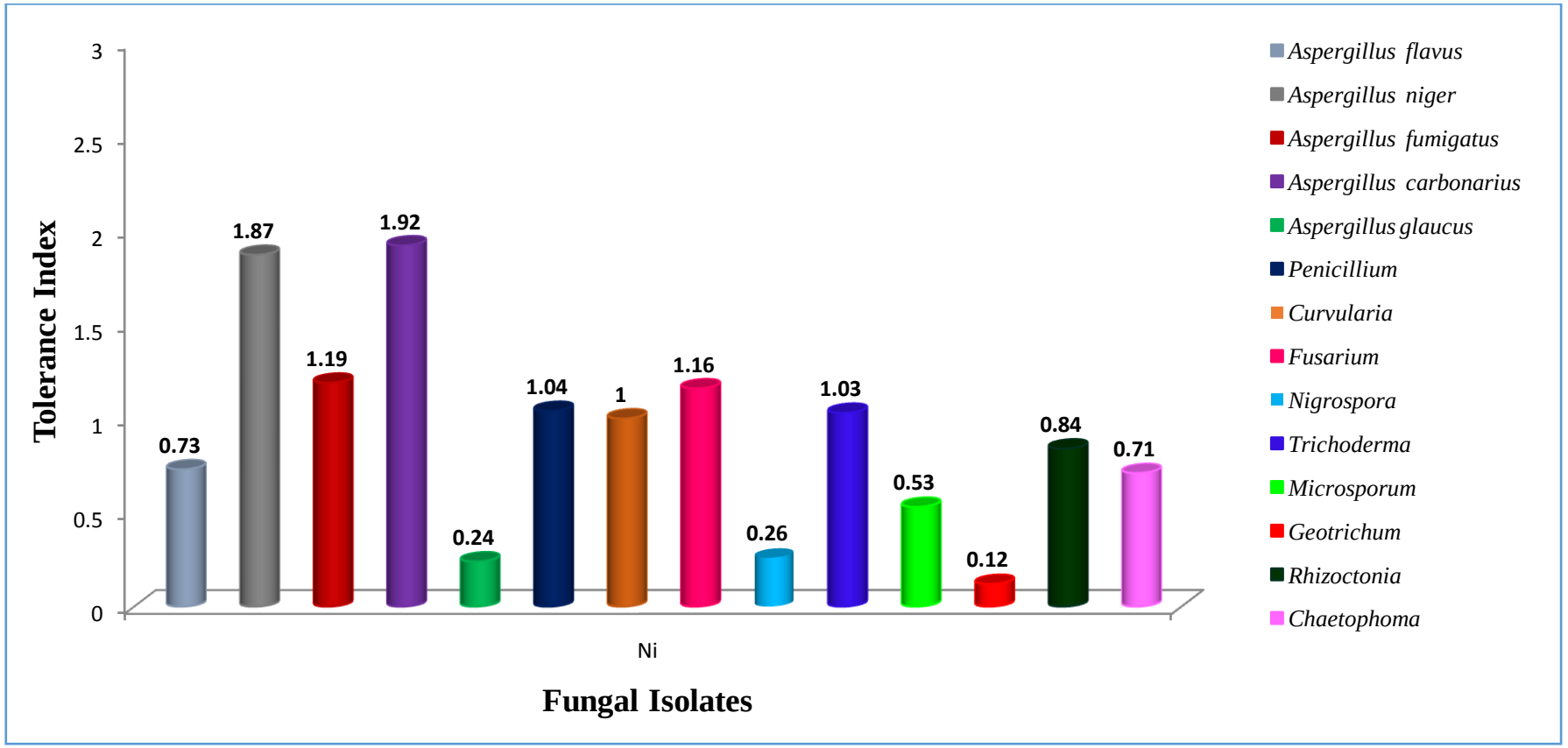


Figure 4.4b: Tolerance Indices of Test Fungal Isolates to Nickel

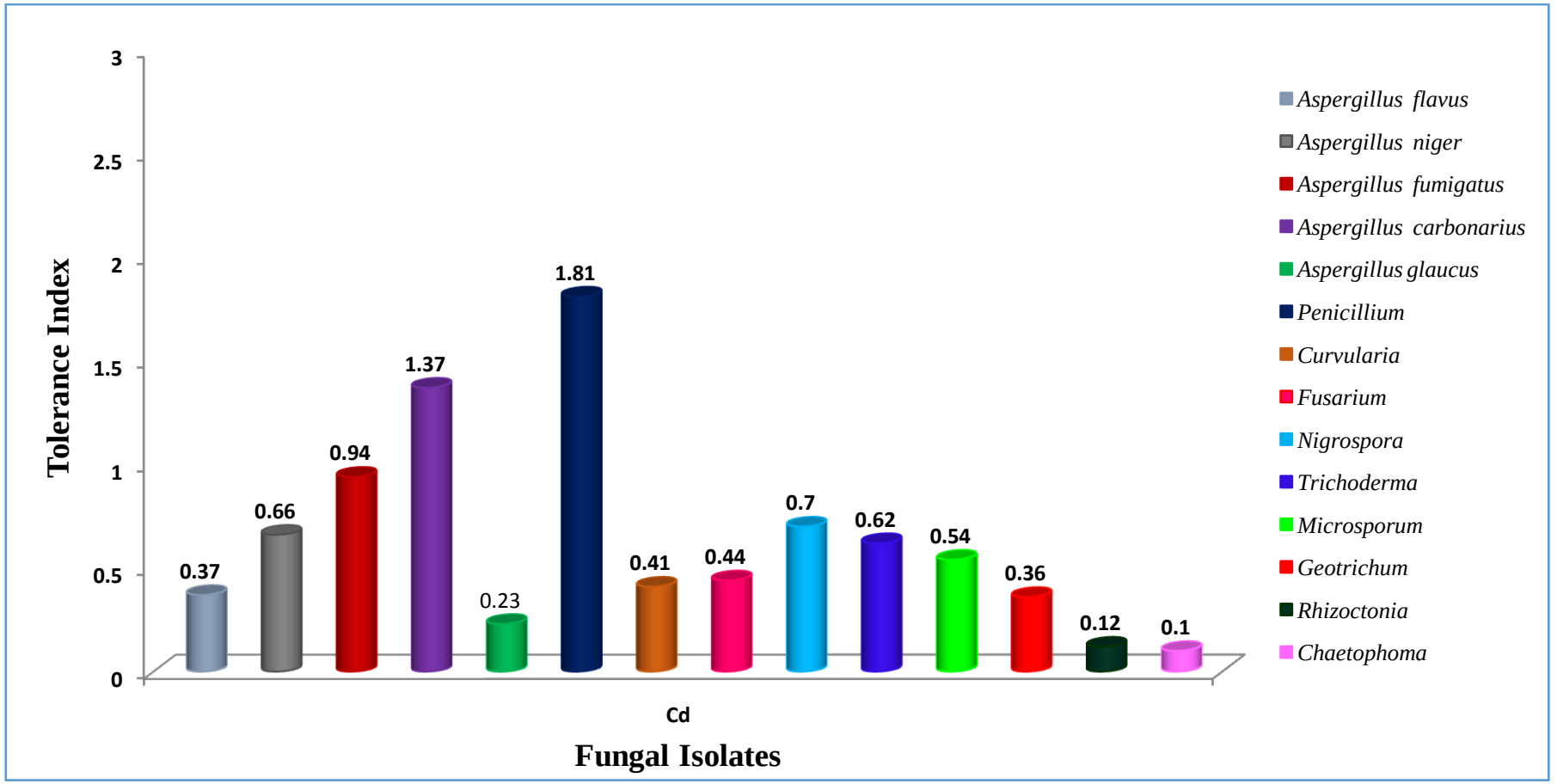


Figure 4.4c: Tolerance Indices of Test Fungal Isolates to Cadmium

4.6 pH and Temperature Optimization

4.6.1 pH optimization

Table 4.7 presents the mean dry biomass yields of the test heavy metal resistant fungal isolates grown at pH 4, 5, 6 and 7. All the *Aspergillus* species grew optimally at pH 6 except *Aspergillus glaucus* which grew optimally at pH 5. The *Penicillium* spp. grew optimally at pH 4 and 6. *Curvularia* spp. and *Fusarium* spp. grew optimally at pH 5. *Nigrospora* spp. grew optimally at pH 6.*Trichoderma, Rhizoctonia* and *Microsporum* species grew optimally at pH 4, pH 5 and pH 6 repectively.

However, *Penicillium, Trichoderma* and *Rhizoctonia* species were found to grow best at pH 4. *Aspergillus, Curvularia, Fusarium, Nigrospora* and *Geotrichum* species grew best at pH 5. On the other hand, *A. flavus, A. niger, A. fumigatus, A. carbonarius, Chaetophoma* and *Microsporum* species grew best at pH 6. None of the isolates were found to be stimulated by pH 7. However, there was significant difference ($P<0.05$) between the means of the biomass yield of the resistant fungal isolates and the test pH

4.6.2 Temperature optimization

Table 4.8 depicts the mean dry biomass yields of the heavy metal resistant fungal isolates grown at temperatures of 25^{o}C, 30^{o}C and 35^{o}C. Only *A. fumigatus* was stimulated at temperature of 25^{o}C. *A. niger, A. carbonarius, A. glaucus, Penicillium* spp., *Curvularia* spp. and *Nigrospora* spp. grew best at temperature of 30^{o}C. On the other hand, *A. flavus, Fusarium* spp., *Chaetophoma* spp., *Trichoderma* spp. and *Geotrichum* spp. grew best at temperature of 35^{o}C. Nevertheless, significant difference ($P<0.05$) exists between the means of the resistant fungal isolates and the varied temperature.

Table 4.7: Mean Biomass Yields of Test Fungal Isolates Grown at varying pH

Fungal Isolates	Dry Biomass Yield±SE (mg)					
	pH 4	pH 5	pH 6	pH 7	F-value	P-value
Aspergillus flavus	79.67±1.72^{bc}	92.33±0.69^{ab}	96.97±7.75^{a}	70.43±1.46^{c}	8.90	0.006
Aspergillus niger	74.80±2.93^{b}	59.80±1.57^{c}	98.50±0.96^{a}	73.33±0.62^{b}	83.70	0.000
Aspergillus fumigatus	73.17±1.84^{b}	73.30±0.57^{b}	93.77±1.56^{a}	73.57±1.95^{b}	42.07	0.000
Aspergillus carbonarius	57.37±2.42^{c}	43.30±1.74^{d}	72.00±1.63^{b}	80.83±2.43^{a}	62.52	0.000
Aspergillus glaucus	87.17±0.67^{b}	93.53±1.92^{a}	52.47±0.32^{d}	58.97±0.63^{c}	357.25	0.000
Penicillium sp.	91.83±2.17^{a}	80.63±0.58^{b}	91.93±2.64^{a}	79.07±1.58^{b}	13.40	0.002
Curvularia sp.	73.10±2.67^{b}	104.07±2.45^{a}	100.03±5.84^{a}	45.17±3.08^{c}	1.26	0.000
Fusarium sp.	72.73±0.50^{b}	96.33±2.30^{a}	44.93±0.32^{c}	44.00±0.51^{c}	452.82	0.000
Chaetophoma sp.	72.17±0.49^{b}	69.43±0.24^{b}	77.10±0.50^{a}	73.87±2.54^{ab}	9.54	0.020
Nigrospora sp.	70.50±0.38^{a}	71.50±0.49^{a}	63.43±1.10^{b}	55.27±1.99^{b}	40.77	0.000
Trichoderma sp.	125.53±11.60^{a}	96.60±0.76^{b}	77.10±1.42^{c}	61.83±1.98^{c}	21.42	0.000
Microsporum sp.	86.87±2.78^{b}	73.77±2.83^{c}	107.23±0.22^{a}	85.07±0.30^{b}	48.77	0.000
Rhizoctonia sp.	148.03±19.93^{a}	92.37±1.47^{b}	91.27±1.60^{b}	62.17±0.15^{b}	12.82	0.002
Geotrichum sp.	78.53±0.34^{c}	107.87±1.72^{a}	92.13±2.96^{b}	72.37±3.36^{c}	43.08	0.000

Means having different superscripted alphabets across row are significantly different at $P \leq 0.05$. Values are expressed as means ± SE (Standard error of means).

Table 4.8: Mean Biomass Yields of Test Fungal Isolates Grown at varying Temperature

Fungal Isolates	Dry Biomass Yield±SE (mg)				
	25°C	30°C	35°C	F-value	P-value
Aspergillus flavus	63.43±1.93^{b}	92.63±1.36^{a}	97.63±1.29^{a}	141.39	0.000
Aspergillus niger	71.77±0.78^{c}	102.77±1.99^{a}	82.90±1.27^{b}	119.60	0.000
Aspergillus fumigatus	98.67±1.60^{a}	87.77±1.59^{b}	67.50±1.15^{c}	116.91	0.000
Aspergillus carbonarius	90.67±0.66^{b}	96.93±0.98^{a}	74.60±2.19^{c}	64.05	0.000
Aspergillus glaucus	89.80±1.22^{b}	98.07±1.01^{a}	78.90±0.78^{c}	89.21	0.000
Penicillium sp.	41.87±0.70^{b}	49.17±1.68^{a}	32.13±1.19^{c}	46.48	0.000
Curvularia sp.	20.33±0.39^{c}	84.00±3.92^{a}	28.47±0.98^{b}	217.95	0.000
Fusarium sp.	53.37±1.43^{c}	69.20±4.40^{b}	92.37±3.15^{a}	36.87	0.000
Chaetophoma sp.	48.50±0.00^{c}	75.10±0.00^{b}	82.00±0.00^{a}	72.31	0.000
Nigrospora sp.	57.80±2.64^{c}	92.73±1.34^{a}	83.57±1.82^{b}	81.36	0.000
Trichoderma sp.	63.67±0.33^{b}	96.87±1.02^{a}	97.63±0.64^{a}	723.56	0.000
Microsporum sp.	21.80±0.90^{c}	63.93±0.56^{a}	51.00±1.22^{b}	535.92	0.000
Rhizoctonia sp.	26.73±1.07^{b}	51.97±1.28^{a}	51.33±1.01^{a}	162.46	0.000
Geotrichum sp.	12.37±0.41^{b}	18.93±0.73^{a}	19.13±0.97^{a}	27.06	0.001

Means having different superscripted alphabets across row are significantly different at P≤0.05. Values are expressed as means ± SE (Standard error of means).^{o}C=Degree centigrade, mg=Milligram

4.7 Heavy Metal Uptake from the Effluent by the Selected Resistant Fungal Isolates

Figures 4.5a-4.5c present heavy metal uptake (mg/g) by the heavy metal resistant fungal isolates. In Figure 4.5a, *Trichoderma* sp. and *Microsporum* sp. recorded equal and highest (0.28mg/g) uptake capacity of lead, followed by *Nigrospora* sp. (0.27mg/g) and *Fusarium* sp. (0.26). *Geotrichum* sp. ranked least (0.01mg/g) in uptake of lead. In Figure 4.5b, *Nigrospora* sp. had the highest (0.14mg/g) nickel uptake. *Fusarium* sp. had a higher nickel uptake value of 0.13mg/g than*A. carbonarius, Microsporum* sp. and consortium of the resistant fungal isolates which had the low and equal nickel uptake of 0.01mg/g each. In Figure 4.5c, *Penicillium* sp. ranked the highest (0.02mg/g) in cadmium uptake. *Curvularia* sp., *Fusarium* sp. and consortium of the resistantfungal isolates had equal cadmium uptake of 0.01mg/g. *Geotrichum* sp. showed no uptake to cadmium.

4.8 Percentage Removal of Heavy Metals from the Effluent by the Resistant Fungal Isolates

The heavy metal resistant fungal isolates were further tested for their ability to remove the heavy metals present in the refinery effluent samples. The results in Figures 4.6a-4.6c depict the percentage removal of Pb, Ni and Cd from the effluent by the resistant fungal isolates. In Figure 4.6a, *A. flavus* and *A. niger* recorded the highest and equal lead removal of 71.29% each. *Geotrichum* sp. recorded the lowest (35.48%) in removal of lead from the effluent samples. Similar pattern was also found in Figure 4.6b which present nickel removal by the isolates. However, *A. niger* and *Geotrichum* sp. ranked the highest (85.06%) and lowest (29.87%) respectively in removing nickel. Figure 4.6c shows the percentage removal of cadmium. *Penicillium* had the highest (85%) percentage removal. Nevertheless, *A. niger* and *A. fumigatus* had equal cadmium removal of 50 % each. This was also observed in *Microsporum* sp. and *Rhizoctonia* sp. with low and equal percentage removal of 15% each. However, there was no Cd removal by Geotrichum sp.

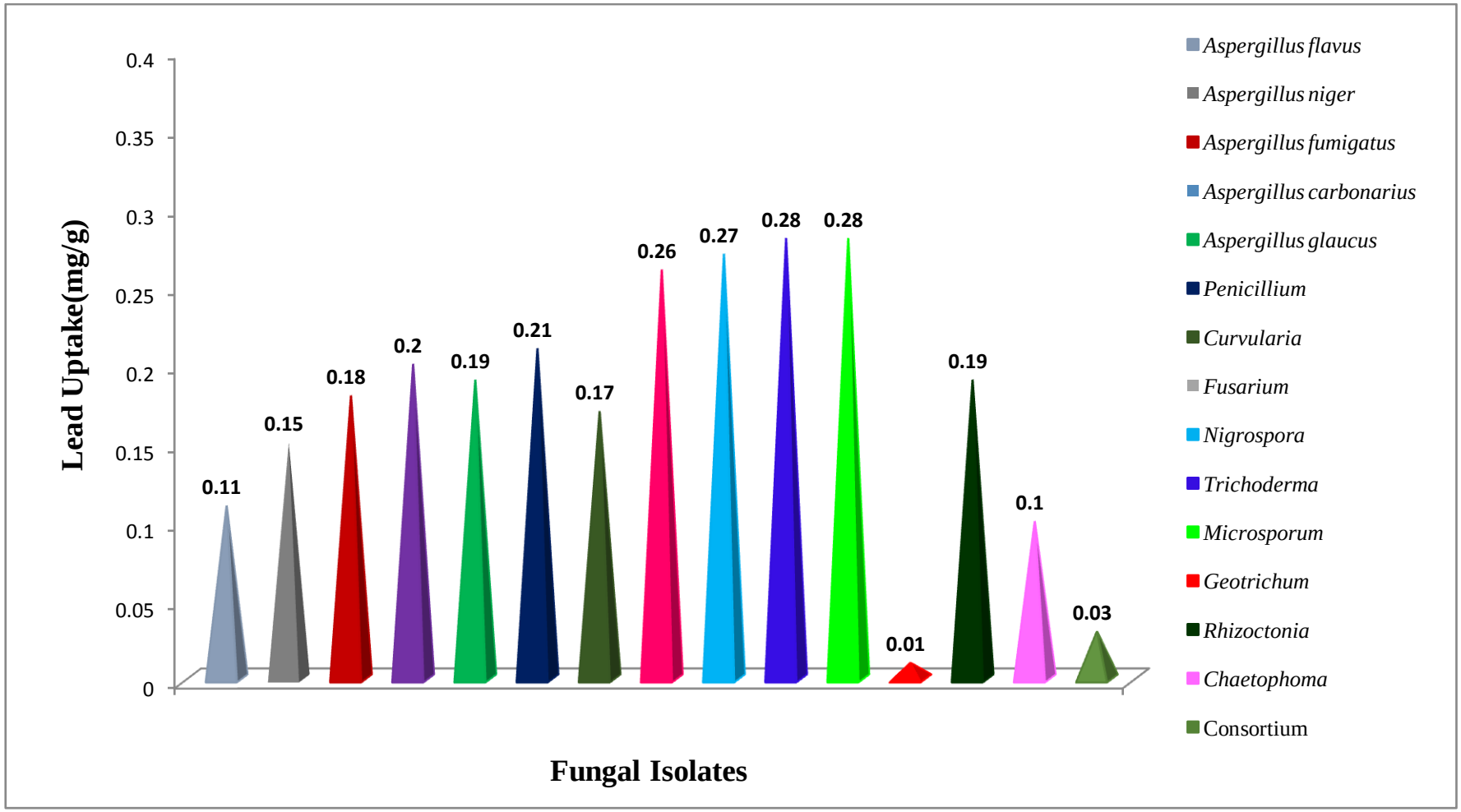


Figure 4.5a: Uptake of Lead by the Test Fungal Isolates

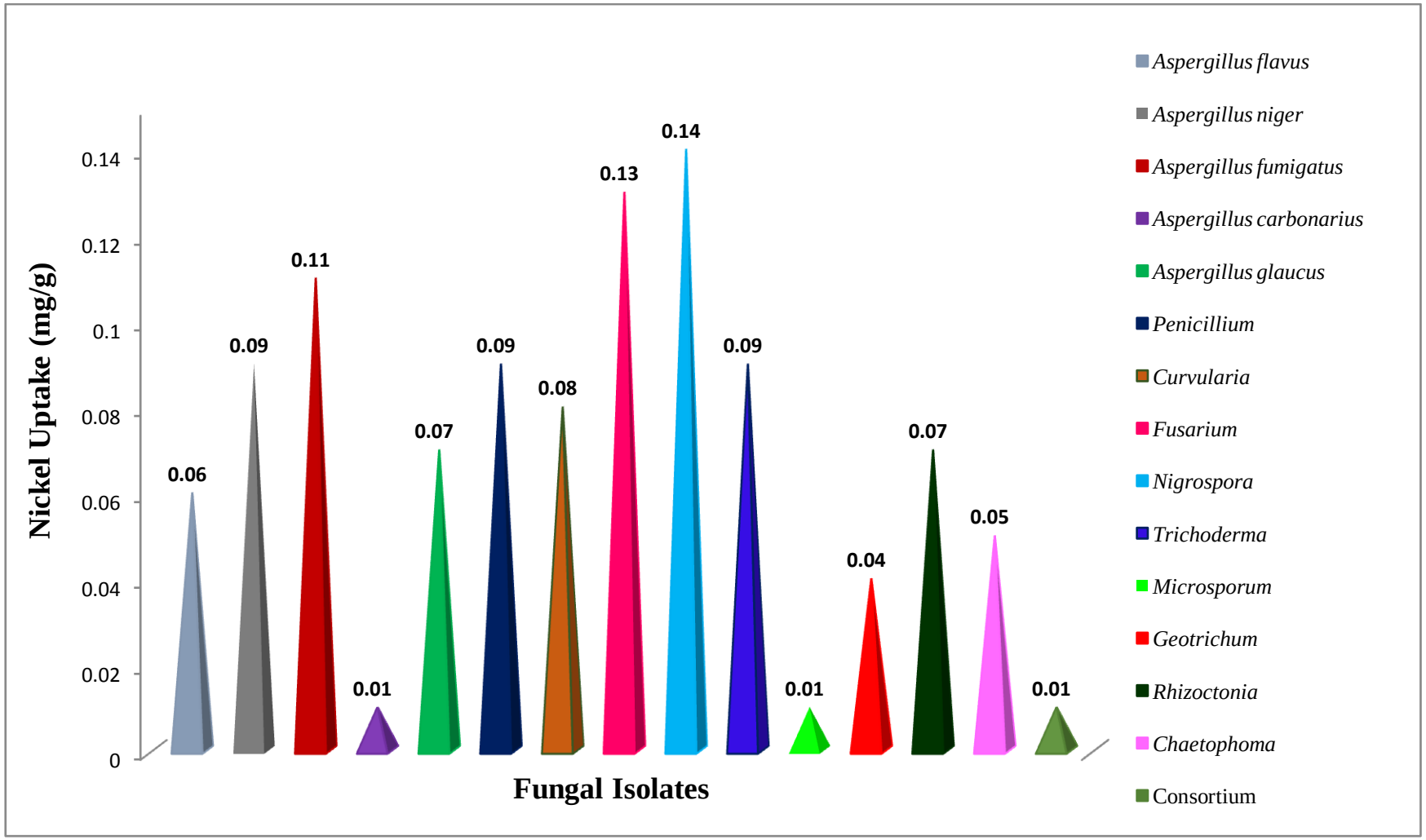


Figure 4.5b: Uptake of Nickel by the Test Fungal Isolates

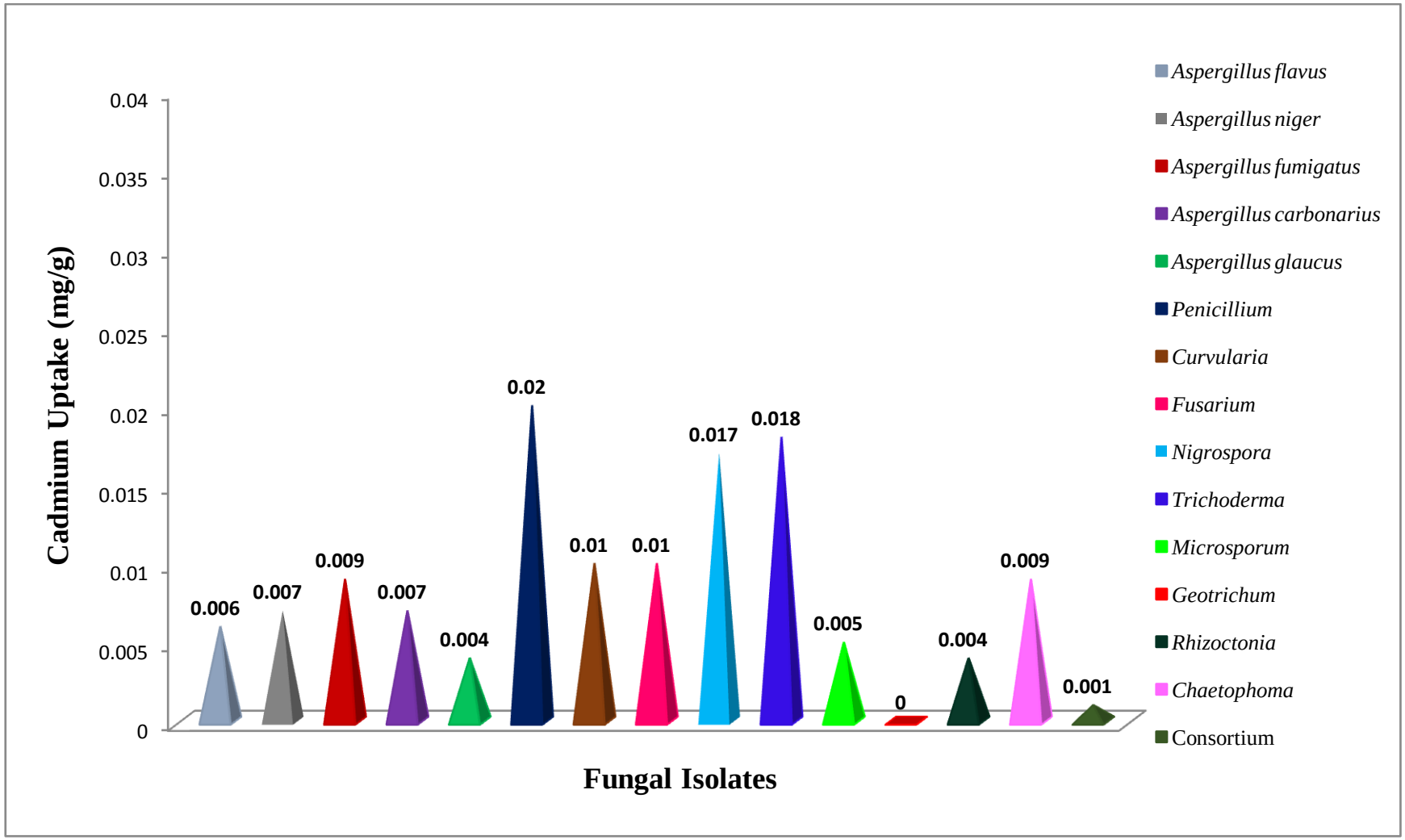


Figure 4.5c: Uptake of Cadmium by the Test Fungal Isolates

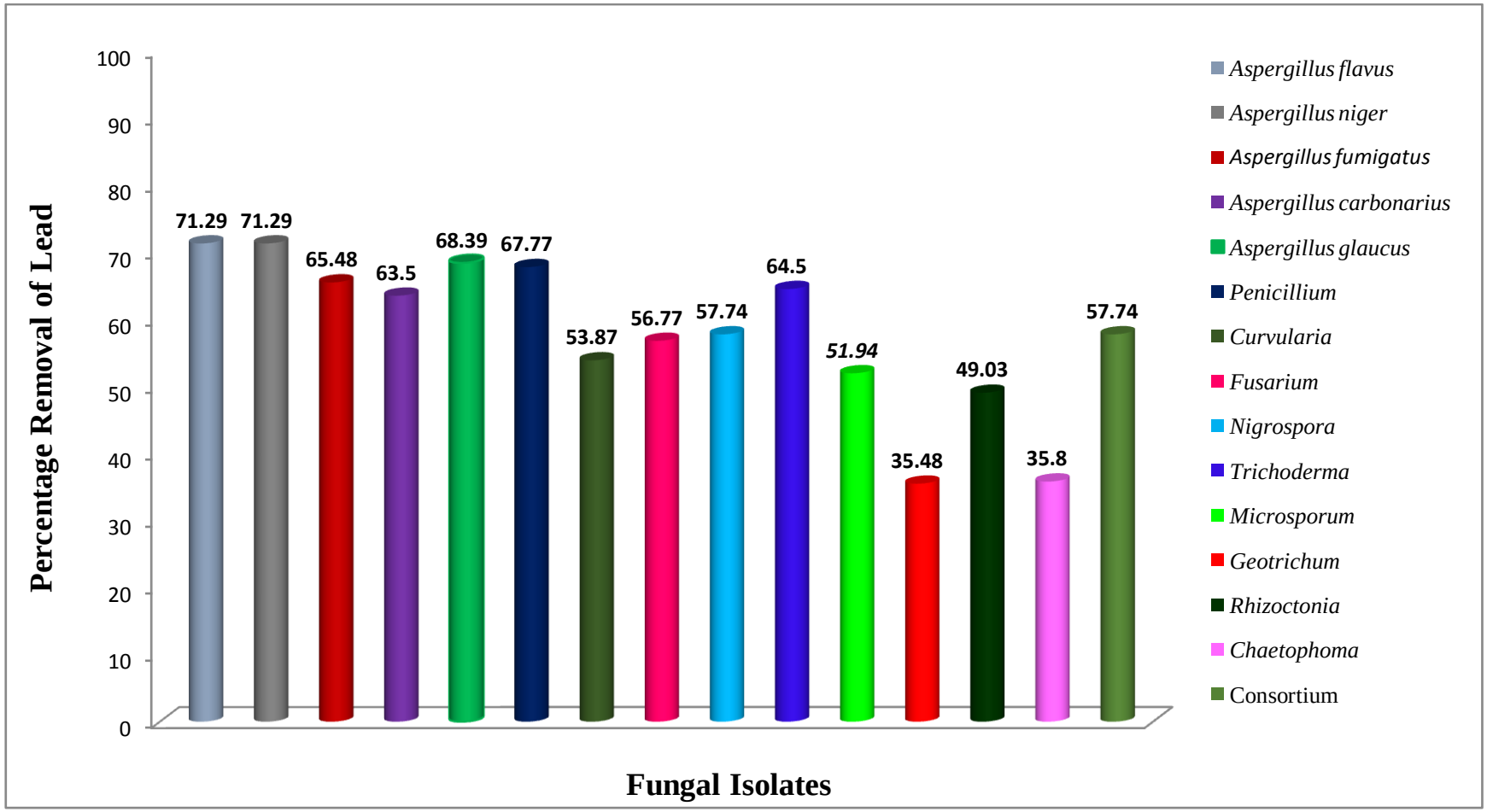


Figure 4.6a: Removal of Lead from Refinery Effluent by the Test Fungal Isolates

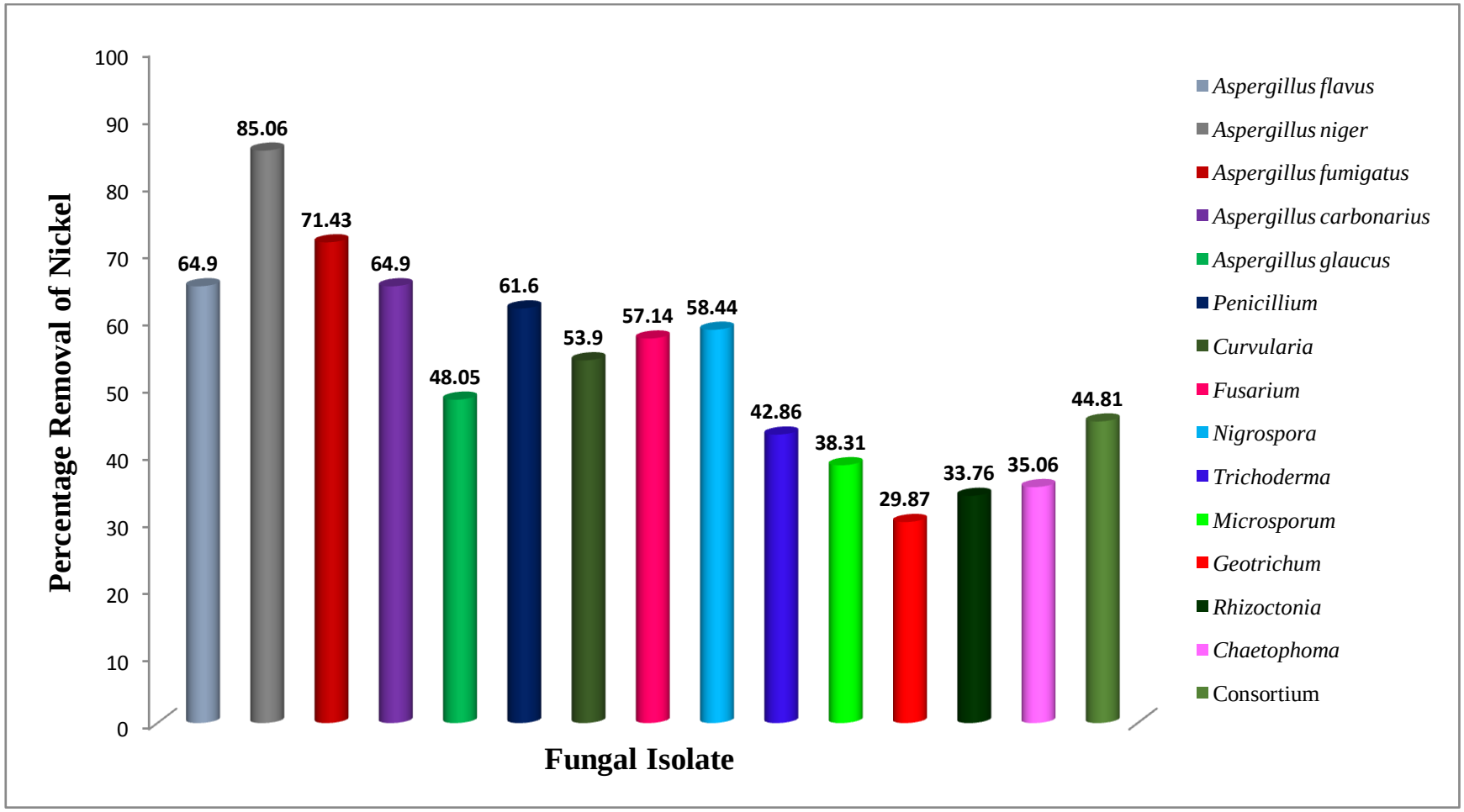


Figure 4.6b: Removal of Nickel from Refinery Effluent by the Test Fungal Isolates

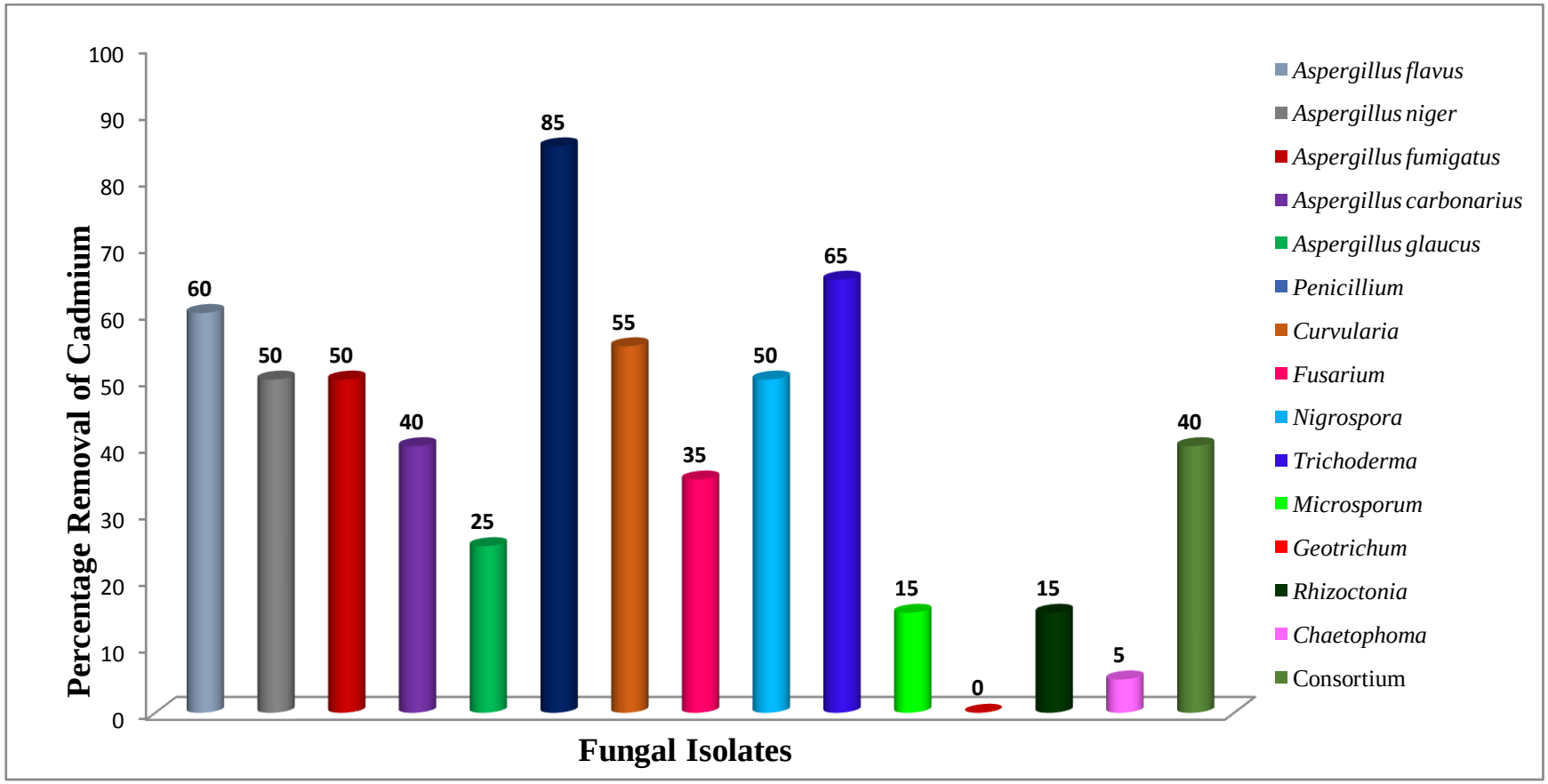


Figure 4.6c: Removal of Cadmium from Refinery Effluent by the Test Fungal Isolates

CHAPTER FIVE

DISCUSSION

5.1 Physicochemical Parameters of the Effluent Samples from the Refinery and Water Samples from Romi River

The physical parameters (temperature, electrical conductivity, and total dissolved solids) were determined. The temperature values of the study site which ranged from 29.53^{o}C at the upstream of Romi River to 36.5^{o}C at the discharge point were within the permissible limits (40^{o}C) of Federal Ministry of Environment (FMENV), Nigeria. However, the high temperature values at the discharge point and downstream of Romi River could be due to increase in rate of reaction and the nature of biological activities, since temperature is an important factor that governs the assimilation capacity of aquatic system (Forstner and Wittlman, 1979). These result (Table 4.1) agrees with the findings of Adefemi and Awokunmi (2009), who had similar temperature and electrical conductivity values.

Electrical conductivity which ranged from 0.18 µS/cm to 1.65 µS/cm along the sampling sites were within the acceptable limit (2 µS/cm) of FMENV. However the electrical conductivity at waste oil retention pond (site B) has the highest acceptable value among the sampling sites. This high value may probably be due to high organic and inorganic compounds from various chemicals used in downstream processes of primary distillation in refinery plant.

The total dissolved solids (TDS) of the samples analyzed at site B (743mg/l), site C (732.33mg/l) and site E (678mg/l) were above the acceptable limits (500mg/l) set by FMENV, upstream of Romi River had the lowest value (81.33mg/l). High TDS value is an indication of high sediments in the sample analyzed. This reduces light penetration

into the aquatic environment thereby decreasing photosynthetic activities. The decreased photosynthetic rate reduces the dissolved oxygen level of waste water by the microorganisms in the samples analyzed (Galadima, 2012).

The chemical parameters of the study sites determined include pH, nitrate, sulphate, phosphate, biological oxygen demand, oil and grease. The pH value of the samples analyzed, revealed that it was in the range of 4.17 to 6.97. The lowest pH (4.17) was observed in the untreated waste water channel (site A) and this was below the permissible limit (6.0-9.0) set by FEMNV. However, the pH of the entire study site was slightly acidic. This low pH may be attributed to acidification of the oil well during drilling completion process and chemicals used during treatment processes (Ayotamunor and Akor, 2002; Thipeswamy *et al*; 2012). This reveals that regardless of the different stages of drilling and treatment operations; acidic effluent discharge could be very hazardous to the environment. The slightly acidic pH recorded in the upstream of Romi River (site D) is probably due to the run off of sulphate based fertilizer from the agricultural areas around the river since the river serves as source of irrigation water to farmers around this region. This is similar to the findings of Atubi (2011) and Lekwot *et al.* (2012). In addition, acidic pH value adversely affects humans, plants and aquatic lives, increases the concentration and toxicity of some dissolved heavy metals and also changes the permeability of soil which results in the pollution of underground resources of water (Galadima, 2012).

On the average, the other chemical parameters analyzed in the study sites were within the acceptable limits by FMENV with their values ranging from lowest to the highest includes; biological oxygen demand (0.87mg/l-3.7 mg/l); Nitrate (18.17mg/l-24.33 mg/l); sulphate (26.33 mg/l-32.17 mg/l); phosphate (0.77mg/l-2.32mg/l) and oil and grease (0.63 mg/l-3.93 mg/l).

5.2 Heavy Metal Content of the Study Site

The contents of Pb, Ni and Cd noted for all the sampling sites ranged from (0.09mg/l to 0.4mg/l); (0.06mg/l to 0.22mg/l) and (0.02mg/l to 0.06mg/l) respectively. These levels of Pb, Ni and Cd are above the acceptable limits set by FMENV, Nigeria. These high levels of Pb, Ni and Cd observed in this study may have originated from feedstock, corrosion products of equipment and pipes, chemical additives and catalyst (nickel sulphate) used in downstream processes (Beddri and Ismail, 2007; Stuart and Milne, 2008).

The high levels of Pb, Ni and Cd in the studied sites could pose serious dangers to public health. This is especially true in situations where large volume of the effluent is continuously released into the environment on a regular basis especially when the effluent is not adequately treated. Where the effluent is released into the soil environment, the content of plant nutrient could be affected resulting in low soil fertility (Dix, 2001). In addition, the poisonous effect of ingesting these metals by aquatic animals, including fishes and their entrance into the food chain cannot be over emphasized. This will in turn cause neurological defects, body organ dysfunctions and anaemia (Ayotamuno and Akor, 2002).

5.3 Mycoflora of the Study Site

One hundred and thirty three (133) fungal isolates were isolated from the studied sites. The observations made in this study suggest that the mycoflora of the raw refinery effluent, effluent stabilization pond and effluent Recipient River is dominated by ten (10) genera. These are *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia*, *Trichoderma, Nigrospora, Microsporum*, *Rhizoctonia*, *Trichophyton* and *Geotrichum* (Table 4.1). This observation strongly indicates that members of these fungal genera have the capacity to

survive and grow in environments that are intensely contaminated with refinery effluents (Edward and White, 1999; Ulfig *et al.,* 2003).

As revealed by the findings (Table 4.2), made in this study, a good number of fungal genera have the capacity to exploit (grow in) the raw or partially treated refinery effluent and in the river that serve as recipient of both. *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. were cosmopolitan to the studied sites while *Nigrospora* and *Chaetophoma* were the least detected genera.Furthermore, *Aspergillus* appeared to constitute the dominant genus making up the fungal flora of the study sites followed by *Penicillium* sp., *Fusarium* sp and *Curvularia* sp. in this order. In such environments, growth of fungi strongly suggests that, the genera present are capable of growth on hydrocarbons (Atlas and Bartha, 1992; Cerniglia, 1992; Tereshina *et al.,* 1999; Ulfig *et al.,* 2003). In addition, fungal flora of such environment needs to be able to withstand the direct toxicity of toxic heavy metal ions often present at high levels in the effluents and sites impacted by the effluents (Willumsen and Karlson, 1997; Tabatabaee *et al.*, 2005; Emoyan *et al.*, 2006; Wuyep *et al.,* 2007; Adewuyi and Olowu, 2012). The ability of the fungi to survive the toxic effects of polycyclic aromatic hydrocarbons (PAHs) (Kari *et al.,* 2003) would be an added ecological advantage.

Furthermore, the ability of the fungi to secrete a wide range of extracellular enzymes into their growth environments have been advanced as an explanation of their capacity to grow on a wide range of carbon sources (Kari *et al.,* 2003). On the other hand, resistance to high levels of toxic heavy metals has been attributed to the capacity of fungi to bioconvert (David and Jay, 2009), bioabsorb (Shaukar *et al.,* 2007; Nilanjana *et al.,* 2008; Ashok *et al.,* 2010) or bioaccumulate (David and Jay, 2009; Martin *et al.,* 2010) the metal ions.

5.4 Resistance to Heavy Metals by the Fungal Isolates

Fifty two (52) out of the one hundred and thirty three (133) fungi isolated from the study sites were resistant to Pb, Ni and Cd (Table 4.5). Most fungi belonging to the genera *Aspergillus, Penicillium,Curvularia,Fusarium,Nigrospora* and *Trichoderma* resident in refinery effluent impacted sites had high level of resistance and tolerance to Pb, Ni and Cd. In addition, members of the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Nigrospora* were the most resistant to Pb and Ni. However, Cd was least resisted by the same genera of fungi (Figures 4.1-4.4).

To survive and grow at the tested concentrations of Pb, Ni and Cd, the test isolates may have developed mechanisms by which the toxicity of the metals is circumvented. Several of such mechanisms have been reported to be employed by fungi growing in environments containing elevated levels of heavy metals. These include; metal exclusion by permeable barriers (Nies and Silver, 1995), extracellular sequestration or accumulation (Teitzel and Mattew, 2003; Nilanjana *et al.,* 2008) and enzymatic modification of the metal ions to less toxic form (Ramarao *et al.,* 1997; Keong 2003). In addition, fungi are reported to possess specific genes for resistance to heavy metal ions. Mostly, these genes are chromosomal but others are acquired through plasmids (Nies, 1999) and the plasmid encoded genes are reported to be induced by the presence of specific metal ions (Rosen, 2002). The genes encoding the synthesis of metal binding proteins such as metallothionein and siderophores are good examples (Valavanidis and Vlachioganni, 2010). These form the basis of bioaccumulation as a mechanism of resistance to heavy metal ions among fungi.

The variation in resistance to Pb, Ni and Cd observed among genera and or species of fungi tested in this study suggest that, members of the same fungal community have different sensitivity to different metals (Keong, 2003; Shazia *et al.,* 2012). As noted in

this study, the same fungi could exhibit different responses to different concentrations of the same metal (Figures 4.1-4.3). Similar observations have been reported by Nies and Silver (1995).

Toxicity of heavy metal depends on the atomic number of metal and hence metal containing high atomic number are more toxic. Among the fungi subjected to heavy metals maximum toxicity was observed in metals containing high atomic number ($_{82}$Pb, $_{48}$Cd, and $_{28}$Ni). This observation is in agreement with earlier results on Cd and Pb accumulation using *Saccharomyces* (Thipeswamy *et al.,* 2012). However, the observations made in this study strongly suggest that, most of the isolates tested have the potential as candidates in mycoremediation of refinery wastes. According to these authors (Nies and Silver, 1995; Keong, 2003; Shazia *et al.,* 2012; Thipeswamy *et al.,* 2012), microbes that exhibit high resistance to high concentrations of soluble heavy metal ions would be selected for use for the treatment of heavy metal contaminated environments.

5.5 pH and Temperature Optimization Studies

pH and temperature are known to be important parameters in enhancing bioaccumulation of heavy metals and degradation of polluted sites. pH and temperature optimization studies were carried out on the resistant fungi (Tables 4.5-4.6) to determine the pH and temperature values that provide an optimal condition for growth and biological activities. The pH and temperature range used for the optimization study was chosen based on the mean pH of the effluent and samples collected from the effluent contaminated sites, as well as from findings reported in literatures (Sharma and Goyal, 2009;Hamza *et al.,* 2012; Palanivelan *et al.,* 2013**;** Tijani, 2014).

5.5.1 pH optimization

Observations on the high biomass yields of the test fungal isolates at varying test pH were similar to the findings of Tijani (2014) who reported that these pH range (5-7), were optimum for the growth and metal uptake of fungi. These acidic pH (5-7) could also explain the high counts of fungal isolates obtained from the study sites because most fungi thrive well at such pH value (Rabah and Ibrahim, 2010). In addition, low pH favours the adsorption of heavy metals by microorganisms. For instance, amino groups of fungal cell wall compounds and other ligands that bind metal ions are protonated at acidic pH values and as well restrict the toxicity of metal cations by repulsive forces (Morale-Barrera *et al.*, 2008; Mythili and Karthikeyan, 2011; Palanivelan *et al.*, 2013). Previous studies also reported that several fungal isolates such as *Fusarium solani*, *F. oxysporum*, *Trichoderma viride* (Rajiv *et al*., 2009) and *Aspergillus niger* (Srivastava and Thakur, 2006) cultured in medium of pH 5.5 also gave a good growth.

5.5.2 Temperature optimization

Temperature is among the parameters that could affect biomass production and is generally considered the most important factor (Delille *et al*., 2004). The observation of high growth of the fungal isolates at 30^{o}C in this research, agrees with the findings reported by researchers (Hamza *et al.*, 2012; Palanivelan *et al*., 2013). Majority of these reports indicate that biosorption capacity of the fungal biomass increases with increase in temperature. However, the common incubation temperature for the growth of fungi such as *A. niger* (Delille *et al*., 2004), *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. (Santos and Linardi, 2004) is taken to be between 25^{o}C and 35°C which agree with this present study having similar organisms as mentioned above. This range of temperature that encourage growth makes these isolates suitable for use in bioremediation.

In addition, the effect of pH and temperature on fungi is based on the activities of the enzymes they produce for their metabolic activities. Also, the stability of the fungal cell wall to heavy metals is pH and temperature dependent. This therefore implies that the use of these organisms as agent of bioremediation would require that such pH and temperature provide optimum efficiency for their activities in contaminated sites. Any slight variations in optimum pH and temperature could disrupt the biological activities of the organisms.

5.6 Capacity of Fungal Isolates to Bioaccumulate and Remove Heavy Metals

5.6.1 Bioaccumulation potential of the test fungi

The heavy metal uptake (bioaccumulation) by the test fungal isolates observed in this study agrees with the reports from previous studies (Kapoor and Viraraghavan, 1995; Viraraghavan and Yan, 2000; Burgess and Almeida, 2010; Joshi *et al.*, 2011).

It was noted in this study that high uptake of lead (Figure 4.5a) was bioaccumulated by *Trichoderma* sp. (0.28mg/g), *Microsporum* sp (0.28mg/g), *Nigrospora* sp. (0.26mg/g), *Penicillium* sp. (0.21mg/g) and *Aspergillus* sp. (0.19mg/g). Nickel (Figure 4.5b) was accumulated by *Nigrospora* sp. (0.14mg/g), *Fusarium* sp. (0.13mg/g) and *Aspergillus* species (0.11mg/g). Cadmium (Figure 4.5c) was accumulated by *Penicillium* sp. (0.02mg/g), *Trichoderma* sp (0.018mg/g) and *Nigrospora* sp. (0.01mg/g).

The accumulation of metal ions from solution by these fungal isolates may be due to active metabolic reactions and passive adsorption which upsets the equilibrium between the mycelium and the dissolved metals, thus causing continuous solubilization of metal ions on the fungal cell wall. Metal accumulation is carried out by initial rapid binding of metal ions on to the negatively charged cell wall followed by penetration in to the cytoplasm and get accumulated inside the cell (Gadd, 1993b; Thipeswamy *et al.*, 2012).

This was as well observed by Kapoor and Viraraghavan (1995), who reported that fungi are able to accumulate heavy metals because their cell walls contain functional groups such as carboxyl, hydroxyl, amine and sulfhydryl among others, which are able to bind metal ions to a greater or less extent. Bioaccumulation of metal ions in fungi may also be attributed to their production of enzymes such as reductases and phosphatases which can solubilize metal phosphates or by production of chelating agents such as siderophores (Morley *et al.*, 1996; Gadd and Sayer, 2000; Alluri *et al.*, 2007).

According to Valavanidis and Vlachogianni (2010), excess metal ions in an organism are actively excreted, compartmentalized in their cells and tissues or metabolically immobilized. Some metal ions which escape these actions become toxic and have adverse effect to the organisms.

Among the filamentous fungi, the genera *Aspergillus* and *Penicillium* have been reported to have high ability to accumulate heavy metals and radionuclides from external environment (Volesky, 1990; Ledin *et al.*, 1996; Keong, 2003). In this present study, the genera *Aspergillus, Penicillium, Trichoderma, Curvularia* and *Fusarium* accumulated high amounts of Pb, Ni and Cd probably due to presence of cysteine rich metallothionein. This protein shows higher affinity towards these metals (Ezzouhri *et al*., 2009; Elkhawaga, 2011).

5.6.2 Removal of Pb, Ni and Cd from the effluent samples by the resistant isolates

The significant heavy metal removal obtained in this study revealed that all the resistant isolates including their consortium were able to remove Pb, Ni and Cd at different percentages. This observation agrees with earlier reports made by Mogollon *et al*. (1998) and Burgess and Almeida (2012).

High percentage removal of Lead (Figure 4.6a), was carried out by *Aspergillus* sp. (71.29%), *Penicillium* sp. (67.77%), *Trichoderma* sp. (64.5%) and *Nigrospora* sp. (57.74%); Nickel (Figure 4.6b) was removed by *Aspergillus* species (85.06%), *Penicillium* sp (61.6%), *Nigrospora* sp. (58.44%), *Fusarium* sp. (57.14%) and *Curvularia* sp. (53.9%); Cadmium removal (Figure 4.6c) was carried out by *Penicillium* sp.(85%), *Trichoderma* sp. (65%), *Aspergillus* sp (60%) and *Curvularia* sp. (55%).

These observations could be attributed to the important role fungi play in detoxification and removal of heavy metals from contaminated sites through physicochemical and biological mechanism by effecting transformation between soluble and insoluble phases between the fungal cells and the heavy metal in contaminated sites (Keong, 2003; Tahir and Naseem, 2007). The observations in heavy metal reduction made in this study was similar to the findings reported by Al Abboud and Alawlaqi (2011), who observed that heterotrophic fungi such as *Trichoderma, Fusarium, Aspergillus* and *Penicillium* were effective in the detoxification of various heavy metals at contaminated sites. Similar reports on heavy metals removal from contaminated sites by fungi have also been published (Wilde and Benneman, 1993; Vajpaye *et al.*, 1995; Wang, 2000; Keong, 2003 and Naja *et al.*, 2005: Thipeswamy *et al.*, 2012).

Similar findings of Tapan *et al.* (2010) also reported that ionic groups of cell wall surface such as glucoronic acids, phosphates and cis-oriented hydroxyl groups act as ligands intracellular sequestration of metals which result in the removal of the metals from aqueous culture medium. Such activities in the case of fungi have been reported in literature during metal bioremoval (Anand *et al*., 2006; Liu *et al.*, 2006; Xiaoxi *et al*., 2010). This study indicates that despite toxic stress of the metals, the isolates had evolved volatilization, extra cellular precipitation, exclusion, cell surface bindings of heavy metals (Shazia *et al.*, 2013).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From the observations made in this study, the following conclusions were drawn:

1. Samples of the refinery effluent and water samples from downstream of the discharge pointcontained higher levels of Pb and Ni than the permissible limits (FEMNV). But, only water samples from downstream of the discharge point contained higher levels of Cd than the permissible limit.
2. The Mycoflora of the investigated sites consisted mainly of *Aspergillus* spp., *Penicillium* spp., *Curvularia* spp., *Fusarium* spp., *Trichoderma* spp., *Nigrospora* spp., *Microsporum* spp., *Chaetophoma* spp., *Rhizoctonia* spp., and *Geotrichum* spp. with *Aspergillus* spp. being the most predominant genus.
3. Of all the genera of fungi isolated from the study sites, *Aspergillus* species appeared to be the most resistant to all tested concentrations of Pb, Ni and Cd.
4. The capacity of the test isolates to function as agents of mycoremediation of the refinery effluent is best at pH 6 and a temperature of 30^{o}C.
5. The fungi isolated from the study sites showed high degree of variability in their capacity to bioaccumulate the test heavy metals in their tissues. *Fusarium, Nigrospora, Trichoderma* and *Microsporum* species had high uptake of lead while *Fusarium* and *Nigrospora* species showed considerably high uptake of Ni, but only *Penicillium, Nigrospora* and *Trichoderma* showed high uptake of Cd.
6. Also, the fungal isolates exhibited variations in their capacity to remove Pb, Ni and Cd ions from the refinery effluent.

6.3 Recommendations

It is recommended that:

1. Fungi with proven capacity to bioaccumulate or remove these heavy metals should be applied in mycoremediation of refinery effluent before it is released into the environment.
2. A facility based on the optimal pH (6) and temperature ($30^{o}C$) should be developed for efficient application of the fungi in the bioremediation of refinery effluent.
3. Regulatory authorities should ensure strict adherence to recommended standard of the levels of heavy metals in effluent released into the environment.
4. Water from the effluent receiving water body should be thoroughly treated to ensure that the levels of these heavy metals are brought to levels recommended for safe use.
5. Communities interacting with rivers that serve as recipient of refinery effluent should be enlightened as to the dangers associated with consumption of untreated water from the rivers.

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APPENDICES

Appendix-I: Satellite Image of the Study Site Showing the Vicinity of KRPC (Google image, 2012)



Appendix-II: Sample Collection from the Study Site



Appendix-III: Media Formulations

Potato CarrotAgar

Composition per liter:
Potatoes…….20.0g
Carrot……20.0g
Agar……..20.0g
Distilled water……...1liter
Source:Atlas (2005)

Potato Dextrose Agar Oxoid (England)

Composition per liter:
Potato infusion from……...200.0g
Dextrose……20.0g
Agar……..15.0g
Distilled water……...1liter
pH 5.6±0.2 at 25^{o}C

Potato Dextrose Agar with 7.5% NaCl

Composition per liter:
Potato infusion from……600.0g
Glucose……..20.0g
NaCl…….....75.0g
Agar……...15.0g
Distilled water……....1liter
pH 5.6±0.2 at 25^{o}C
Source:Atlas (2005)

Potato Dextrose Broth

Composition per liter:
Potato infusion from……200.0g
Dextrose……20.0g
Distilled water……1liter
pH 5.6±0.2 at 25^{o}C
Source: Atlas (2005)

Appendix-IV: Display of the Fungal Isolates from the Study Site





Appendix- V: Heavy metal Salt Formulations

Nickel Sulphate ($NiSO_4.6H_2O$) Koch-light laboratories, Coinbrook Berks England

Molecular weight (M.W) = 262.85

Composition	%W/W
Purity	99.00
Nickel (Ni)	23.0
Chloride (Cl)	0.01
Sulphate (SO_4)	0.50
Iron (Fe)	0.02
Nitrate (NO_3)	0.001

Lead Acetate ($(CH_3.COO)_2Pb.3H_2O$) Koch-light laboratories, Coinbrook Berks England

M.W= 379.33g/mol

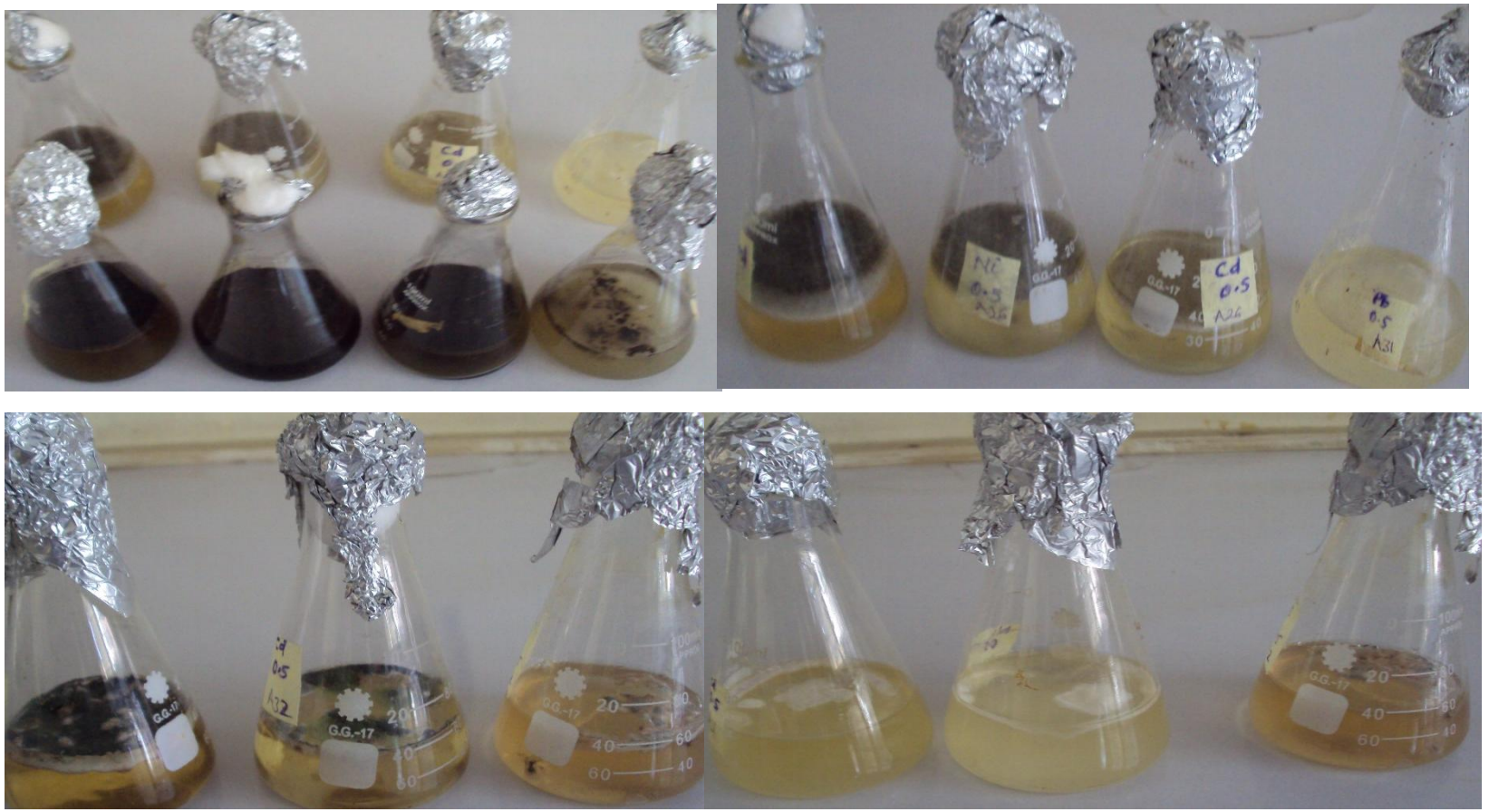
Composition	%W/W
Purity	99.51
Calcium (Ca)	0.005
Potassium (K)	0.002
Iron (Fe)	0.001
Sodium (Na)	0.005
Nitrate (NO_3)	0.005
Insoluble matter	0.0005

Cadmium Chloride (CdCl) Burgoynye, Mumbia, India

M.W= 183.32g/mol

Composition	%W/W
Purity	95
Sulphate	0.02
Ammonium	0.01

Appendix- VI: Set up on Bioaccumulation and Removal of Heavy Metals



Appendix-VII: Harvested Dried Biomass Yield of the Fungal Isolates on Pre-weighed Whatman Filter paper



Appendix-VIII: Heavy Metal Analysis of Samples Using Atomic Absorption Spectrophotometer (AAS 240)



